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## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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<b>(21) International Application Number:</b> PCT/US98/22821 <b>(22) International Filing Date:</b> 28 October 1998 (28.10.98) <b>(30) Priority Data:</b> 08/961,469 31 October 1997 (31.10.97) US <b>(63) Related by Continuation (CON) or Continuation-in-Part (CIP) to Earlier Application</b> US 08/961,469 (CIP) Filed on 31 October 1997 (31.10.97) <b>(71) Applicant (for all designated States except US):</b> ISIS PHARMACEUTICALS, INC. [US/US]; Carlsbad Research Center, 2292 Faraday Avenue, Carlsbad, CA 92008 (US). <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> HARDEE, Gregory, E. [US/US]; 17407 La Brisa, Rancho Santa Fe, CA 92067 (US). GEARY, Richard, S. [US/US]; 3352 Avenida Nieve, Carlsbad, CA 92009 (US). LEVIN, Arthur [US/US]; 15951 Avenida Calma, Rancho Santa Fe, CA 92091 (US). TEMPLIN, Michael, V. [US/US]; 4596 Essex Court, Carlsbad, CA 92008 (US). HOWARD, Randy [US/US]; 1832 Avenida Josefa, Encinitas, CA 92024 (US). MEHTA, Rahul, C.		[IN/US]; 695 Shenandoah Avenue, San Marcos, CA 92069 (US). <b>(74) Agents:</b> LICATA, Jane, Massey et al.; Law Offices of Jane Massey Licata, 66 E. Main Street, Marlton, NJ 08053 (US). <b>(81) Designated States:</b> AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). <b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> LIPOSOMAL OLIGONUCLEOTIDE COMPOSITIONS FOR MODULATING ras GENE EXPRESSION <b>(57) Abstract</b> Pharmaceutical compositions comprising liposomes containing antisense oligonucleotides are provided for the modulation of expression of the human ras gene in both the normal (wildtype) and activated (mutant) forms.		

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**LIPOSOMAL OLIGONUCLEOTIDE COMPOSITIONS  
FOR MODULATING ras GENE EXPRESSION**

**FIELD OF THE INVENTION**

This invention relates to pharmaceutical compositions  
5 comprising liposomes containing one or more antisense  
oligonucleotides. The antisense oligonucleotides contained  
within liposomes are from about 8 to about 30 nucleotides in  
length, are targeted to a nucleic acid encoding a human  
wildtype or mutant ras sequence and are capable, individually  
10 and/or collectively, of modulating ras expression. In another  
embodiment, the liposomes of the invention contain (a) one or  
more such antisense oligonucleotides and (b) one or more  
chemotherapeutic compounds which do not function by an  
antisense mechanism.

**15 BACKGROUND OF THE INVENTION**

Alterations in the cellular genes which directly or  
indirectly control cellular growth (proliferation) and  
differentiation are considered to be causative events leading  
to the development of tumors and cancers (see, generally,  
20 Weinberg, *Sci. American* 275:62, 1996). There are many  
families of genes presently implicated in human tumor  
formation. Members of one such family, the ras gene family,  
are frequently found to be mutated in human tumors. In their  
normal state, proteins produced by the ras genes are thought  
25 to be involved in normal cell growth and maturation. Mutation

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of the ras gene, causing an amino acid alteration at one of three critical positions in the protein product, results in conversion to a form which is implicated in tumor formation. A gene having such a mutation is said to be "activated." It is thought that such a point mutation leading to ras activation can be induced by carcinogens or other environmental factors. Over 90% of pancreatic adenocarcinomas, about 50% of adenomas and adenocarcinomas of the colon, about 50% of adenocarcinomas of the lung and carcinomas of the thyroid, and a large fraction of malignancies of the blood such as acute myeloid leukemia and myelodysplastic syndrome have been found to contain activated ras genes. Overall, some 10 to 20% of human tumors have a mutation in one of the three ras genes (H-ras, K-ras and N-ras).

It is presently believed that inhibiting expression of activated cancer-associated genes in a particular tumor cell might force the cell back into a more normal growth habit. For example, Feramisco et al. (*Nature*, 314:639, 1985) demonstrated that cells transformed to a malignant state with an activated ras gene slow their rate of proliferation and adopt a more normal appearance when microinjected with an antibody which binds to the protein product of the ras gene. This has been interpreted as support for the involvement of the product of the activated ras gene in the uncontrolled growth typical of cancer cells.

The H-ras gene has recently been implicated in a serious cardiac arrhythmia called long Q-T syndrome, a hereditary condition which often causes sudden death if treatment is not given immediately. Frequently there are no symptoms prior to the onset of the erratic heartbeat. Whether the H-ras gene is precisely responsible for long Q-T syndrome is unclear. However, there is an extremely high correlation between inheritance of this syndrome and the presence of a

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particular variant of the chromosome 11 region surrounding the H-ras gene. Therefore, the H-ras gene is a useful indicator of increased risk of sudden cardiac death due to the long Q-T syndrome.

5           There is a great desire to provide compositions of matter which can modulate the expression of the ras gene, and particularly to provide compositions of matter which specifically modulate the expression of the activated form of the ras gene. Inhibition of K-ras gene expression has been  
10 accomplished using retroviral vectors or plasmid vectors which express a 2-kilobase segment of the K-ras gene RNA in antisense orientation (Mukhopadhyay et al., *Cancer Research* 51:1744, 1991; PCT Patent Application PCT/US92/01852 (WO 92/15680); Georges et al., *Cancer Research*, 53:1743, 1993).

15           Antisense oligonucleotide inhibition of expression has proven to be a useful tool in understanding the role(s) of various cancer-associated gene families. Antisense oligonucleotides are small oligonucleotides which are complementary to the "sense" (coding strand) of a given gene,  
20 and are thus also complementary to, and thus able to stably and specifically hybridize with, the mRNA transcript of the gene. Holt et al. (*Mol. Cell Biol.* 8:963, 1988) state that antisense oligonucleotides designed to hybridize specifically with (i.e., "targeted to") mRNA transcripts of the c-myc gene  
25 inhibit proliferation and induce differentiation when added to cultured HL60 leukemic cells. Anfossi et al. (*Proc. Natl. Acad. Sci.* 86:3379, 1989) state that antisense oligonucleotides targeted to the c-myb gene inhibit proliferation of human myeloid leukemia cell lines. Wickstrom  
30 et al. (*Proc. Nat. Acad. Sci.* 85:1028, 1988) state that expression of the protein product of the c-myc gene and proliferation of HL60 cultured leukemic cells are both inhibited by antisense oligonucleotides hybridizing specifically with c-myc mRNA.

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With specific regard to oligonucleotides having ras sequences, United States Patent No. 4,871,838 to Bos *et al.* discloses oligonucleotides complementary to a mutation in codon 13 of N-ras to detect this mutation. Helene and co-workers have reported the selective inhibition of activated (codon 12 G-T transition) H-ras mRNA expression using a 9-mer phosphodiester linked to an acridine intercalating agent and/or a hydrophobic tail; this compound displayed selective targeting of mutant ras message in both Rnase H and cell proliferation assays at low micromolar concentrations (Saison-Behmoaras *et al.*, *EMBO J.* 10:1111, 1991). Chang *et al.* (*Biochemistry* 30:8283, 1991) disclose selective targeting of a mutant H-ras message, specifically, H-ras codon 61 containing an A-T transversion, with an 11-mer methylphosphonate oligonucleotide or its psoralen derivative. These compounds, which required concentrations of 7.5-150  $\mu$ M for activity, were shown by immunoprecipitation to selectively inhibit mutant p21<sup>H-ras</sup> expression relative to wildtype p21<sup>H-ras</sup>.

Although it has been recognized that antisense oligonucleotides have great therapeutic potential, there remains a long-felt need for pharmaceutical compositions and methods that could positively alter the *in vivo* stability, concentration, and distribution of such oligonucleotides. Enhanced biostability of antisense oligonucleotides in a mammal would generally be preferred for improved delivery of the oligonucleotide to its intended target tissue(s) with potentially less frequent dosing. For antisense oligonucleotides targeted to oncogenic molecules, enhanced distribution to tumor tissues would be preferred.

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**OBJECTS OF THE INVENTION**

It is an object of the invention to provide liposomes containing one or more antisense oligonucleotides and pharmaceutical compositions comprising such liposomes, wherein  
5 the antisense oligonucleotides contained within the liposomes are from about 8 to about 30 nucleotides in length, are targeted to a nucleic acid encoding a human ras sequence and are capable, either individually or collectively, of modulating ras expression.

10 It is another object of the invention to provide liposomes containing one or more antisense oligonucleotides, and pharmaceutical compositions comprising such liposomes, wherein the antisense oligonucleotides contained within the liposomes are from about 8 to about 30 nucleotides in length,  
15 are targeted to a nucleic acid encoding an activated (mutant) human ras sequence and are capable, either individually or collectively, of modulating the expression of the activated form of the ras gene.

It is a further object of the invention to provide  
20 liposomes containing (a) one or more antisense oligonucleotides being from about 8 to about 30 nucleotides in length, targeted to a nucleic acid encoding either a wildtype or mutant human ras sequence which are capable, either individually or collectively, of modulating ras  
25 expression and (b) one or more chemotherapeutic compounds which do not function by an antisense mechanism.

An additional object of the invention is to provide liposome-based pharmaceutical compositions which inhibit the hyperproliferation of cells, including cancerous cells.  
30 Methods of inhibiting the hyperproliferation of cells, including cancerous cells, are also an object of this invention.

A further object of this invention is to provide methods of treatment of, and liposome-based pharmaceutical

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compositions for, conditions arising due to mutation of the gene from the wildtype to a mutant, activated form of the ras gene.

#### SUMMARY OF THE INVENTION

5           In accordance with the present invention liposomes containing one or more antisense oligonucleotides and pharmaceutical compositions comprising such liposomes are provided, wherein the antisense oligonucleotides contained within the liposomes are from about 8 to about 30 nucleotides  
10 in length, are targeted to a nucleic acid encoding a human ras sequence and are capable, either individually or collectively, of modulating ras expression.

          Also provided are liposomes containing one or more antisense oligonucleotides, and pharmaceutical compositions  
15 comprising such liposomes, wherein the antisense oligonucleotides contained within the liposomes are from about 8 to about 30 nucleotides in length, are targeted to a nucleic acid encoding an activated (mutant) human ras sequence and are capable, either individually or collectively, of modulating  
20 the expression of the activated form of the ras gene.

          Further provided are liposomes containing (a) one or more antisense oligonucleotides being from about 8 to about 30 nucleotides in length, targeted to a nucleic acid encoding either a wildtype or mutant human ras sequence which are  
25 capable, either individually or collectively, of modulating ras expression and (b) one or more chemotherapeutic compounds which do not function by an antisense mechanism.

          Liposome-based pharmaceutical compositions which inhibit the hyperproliferation of cells, including cancerous  
30 cells, are provided. Methods of inhibiting the hyperproliferation of cells, including cancerous cells, are also provided.

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Methods of treatment of, and liposome-based pharmaceutical compositions for, conditions arising due to mutation of the gene from the wildtype to a mutant, activated form of the ras gene are also provided herein.

## 5 BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the time course of the clearance of ISIS 2503 and oligonucleotide metabolites from blood. Each point is represented as the mean (symbol)  $\pm$  standard deviation (n=1-8). Nonlinear regression was performed using a one  
10 compartment model (solid line). Symbols: ●, observed blood concentration for liposomal oligonucleotide formulation; the solid line indicates the predicted blood concentration for liposomal oligonucleotide formulation; ▲, observed blood concentration for saline formulation of ISIS 2503.

15 Figure 2 shows the distribution and kinetics of full-length ISIS 2503 in monkey tissues at time points following a single 10 mg/kg intravenous infusion of ISIS 2503 encapsulated in sterically stabilized liposomes in the indicated tissues ("A & I Lymph" = axillary and inguinal lymph  
20 nodes, combined; "M & M Lymph" = mesenteric and mandibular lymph nodes, combined). Symbols: X, axillary and inguinal lymph nodes, combined; ▲, mesenteric and mandibular lymph nodes, combined; ●, hand skin; ◆, back skin.

Figure 3 shows the distribution and kinetics of full-  
25 length ISIS 2503 in monkey tissues at time points following a single 10 mg/kg intravenous infusion of ISIS 2503 encapsulated in sterically stabilized liposomes in the indicated tissues ("A & I Lymph" = axillary and inguinal lymph nodes, combined; "M & M Lymph" = mesenteric and mandibular  
30 lymph nodes, combined). Symbols: X, liver; ▲, spleen; ●, kidney cortex; ◆, kidney medulla.

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Figure 4 shows the distribution and kinetics of total oligonucleotide in monkey tissues at time points following a single 10 mg/kg intravenous infusion of ISIS 2503 encapsulated in sterically stabilized liposomes in the indicated tissues  
5 ("A & I Lymph" = axillary and inguinal lymph nodes, combined; "M & M Lymph" = mesenteric and mandibular lymph nodes, combined). Symbols: X, axillary and inguinal lymph nodes, combined; ▲, mesenteric and mandibular lymph nodes, combined; ●, hand skin; ♦, back skin.

10 Figure 5 shows the distribution and kinetics of total oligonucleotide in monkey tissues at time points following a single 10 mg/kg intravenous infusion of ISIS 2503 encapsulated in sterically stabilized liposomes in the indicated tissues ("A & I Lymph" = axillary and inguinal lymph nodes, combined;  
15 "M & M Lymph" = mesenteric and mandibular lymph nodes, combined). Symbols: X, liver; ▲, spleen; ●, kidney cortex; ♦, kidney medulla.

Figures 6A and 6B are representative electropherograms of a liposomal (Figure 6A) or saline  
20 formulation (Figure 6B) of ISIS 2503 in monkey blood samples. Samples were taken 60 hours (a) after an 0.5 hour infusion or (b) 1 hour after initiation of a 2-hour infusion of 10 mg/kg of the respective formulations. Peaks corresponding to ISIS 2503 and various metabolites (arrows) or to an internal  
25 standard (T<sub>27</sub>, a 27-mer phosphorothioate oligodeoxythymidine) are indicated.

Figures 7A and 7B are representative electropherograms of a liposomal (Figure 7A) or saline  
30 formulation (Figure 7B) of ISIS 2503 in monkey kidney cortex samples. Samples were taken 60 hours (a) after an 0.5 hour infusion or (b) 48 hours after the last 2-hour infusion of 14 total doses administered every other day of 10 mg/kg of the respective formulations. Peaks corresponding to ISIS 2503 and



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various metabolites (arrows), including a suspected (n+1) species (see Examples), or to an internal standard (T<sub>27</sub>, a 27-mer phosphorothioate oligodeoxythymidine) are indicated.

#### DETAILED DESCRIPTION OF THE INVENTION

5 Malignant tumors develop through a series of stepwise, progressive changes that lead to the loss of growth control characteristic of cancer cells, i.e., continuous unregulated proliferation, the ability to invade surrounding tissues, and the ability to metastasize to different organ  
10 sites. Carefully controlled *in vitro* studies have helped define the factors that characterize the growth of normal and neoplastic cells and have led to the identification of specific proteins that control cell growth and differentiation. In addition, the ability to study cell  
15 transformation in carefully controlled, quantitative *in vitro* assays has led to the identification of specific genes capable of inducing the transformed cell phenotype. Such cancer-associated genes are believed to acquire transformation-inducing properties through mutations leading to changes in  
20 the regulation of expression of their protein products. In some cases such changes occur in non-coding DNA regulatory domains, such as promoters and enhancers, leading to alterations in the transcriptional activity of cancer associated genes, resulting in over- or under-expression of  
25 their gene products. In other cases, gene mutations occur within the coding regions of cancer associated genes, leading to the production of altered gene products that are inactive, overactive, or exhibit an activity that is different from the normal (wild-type) gene product.

30 Many cellular cancer associated gene families have been identified and categorized on the basis of their subcellular location and the putative mechanism of action of their protein products. The ras genes are members of a gene

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family which encode related proteins that are localized to the inner face of the plasma membrane. Ras proteins have been shown to be highly conserved at the amino acid level, to bind GTP with high affinity and specificity, and to possess GTPase  
5 activity (for a review, see Downward, *Trends Biochem. Sci.* 15:469, 1990). Although their cellular function(s) is(are) unknown, the biochemical properties of the ras proteins, along with their significant sequence homology with a class of signal-transducing proteins known as GTP binding proteins, or  
10 G proteins, suggest that ras gene products play a fundamental role in basic cellular regulatory functions relating to the transduction of extracellular signals across plasma membranes. The ras gene product, p21<sup>ras</sup>, interacts with a variety of known and proposed cellular effectors (for a review, see Marshall,  
15 *Trends Biochem. Sci.* 18:250, 1993)

Three ras genes, designated H-ras, K-ras, and N-ras, have been identified in the mammalian genome. Mammalian ras genes acquire transformation-inducing properties by single point mutations within their coding sequences. Mutations in  
20 naturally occurring ras genes have been localized to codons 12, 13, and 61. The sequences of H-ras, K-ras and N-ras are known (Capon et al., *Nature* 302:33, 1983; Kahn et al., *Anticancer Res.* 7:639, 1987; Hall and Brown, *Nucleic Acids Res.* 13:5255, 1985). The most commonly detected activating  
25 ras mutation found in human tumors is in codon 12 of the H-ras gene in which a base change from GGC to GTC results in a glycine-to-valine substitution in the GTPase regulatory domain of the ras protein product (Tabin et al., *Nature*, 300:143, 1982; Reddy et al., *Nature* 300:149, 1982; Taparowsky et al.,  
30 *Nature* 300:762, 1982). This single amino acid change is thought to abolish normal control and/or function of p21<sup>H-ras</sup>, thereby converting a normally regulated cell protein to one that is continuously active. It is believed that such

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deregulation of normal ras protein function is responsible for the transformation from normal to malignant growth.

The present invention provides pharmaceutical compositions comprising liposomes containing one or more antisense oligonucleotides, wherein the antisense oligonucleotides contained within the liposomes are from about 8 to about 30 nucleotides in length, more preferably from about 8 to about 30 nucleotides in length, are targeted to a nucleic acid encoding a human wildtype or mutant ras sequence and are capable, individually and/or collectively, of modulating ras expression. In another embodiment, compositions of the invention comprise sterically stabilized liposomes containing (a) one or more such antisense oligonucleotides and (b) one or more chemotherapeutic compounds which do not function by an antisense mechanism. The remainder of the Detailed Description relates in more detail to (1) the oligonucleotides of the invention, (2) their bioequivalents, (3) sterically stabilized liposomes, (4) chemotherapeutic agents that can be combined with antisense oligonucleotides targeted to H-ras in the context of the liposomes of the invention and (5) administration of pharmaceutical compositions comprising the liposomal oligonucleotide compositions of the invention.

**1. Oligonucleotides:** In the context of this invention, the term "oligonucleotide" refers to an oligomer or polymer of ribonucleic acid or deoxyribonucleic acid. This term includes oligonucleotides composed of naturally-occurring nucleobases, sugars and covalent intersugar (backbone) linkages as well as oligonucleotides having non-naturally-occurring portions which function similarly. Such modified or substituted oligonucleotides are often preferred over native forms because of desirable properties such as, for example, enhanced cellular uptake, enhanced binding to target and increased stability in the presence of nucleases.

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An oligonucleotide is a polymer of a repeating unit generically known as a nucleotide. The oligonucleotides in accordance with this invention preferably comprise from about 8 to about 30 nucleotides. An unmodified (naturally occurring) nucleotide has three components: (1) a nitrogen-containing heterocyclic base linked by one of its nitrogen atoms to (2) a 5-pentofuranosyl sugar and (3) a phosphate esterified to one of the 5' or 3' carbon atoms of the sugar. When incorporated into an oligonucleotide chain, the phosphate of a first nucleotide is also esterified to an adjacent sugar of a second, adjacent nucleotide via a 3'-5' phosphate linkage. The "backbone" of an unmodified oligonucleotide consists of (2) and (3), that is, sugars linked together by phosphodiester linkages between the 5' carbon of the sugar of a first nucleotide and the 3' carbon of a second, adjacent nucleotide. A "nucleoside" is the combination of (1) a nucleobase and (2) a sugar in the absence of (3) a phosphate moiety (Kornberg, A., *DNA Replication*, W.H. Freeman & Co., San Francisco, 1980, pages 4-7). The backbone of an oligonucleotide positions a series of bases in a specific order; the written representation of this series of bases, which is conventionally written in 5' to 3' order, is known as a nucleotide sequence.

The oligonucleotides used in accordance with this invention may be conveniently and routinely made through the well-known technique of solid phase synthesis. Equipment for such synthesis is sold by several vendors including Applied Biosystems (Foster City, CA). Any other means for such synthesis may also be employed, however, the actual synthesis of the oligonucleotides are well within the talents of the routineer. It is also well known to use similar techniques to prepare other oligonucleotides such as the phosphorothioates and alkylated derivatives.

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Oligonucleotides may comprise nucleotide sequences sufficient in identity and number to effect specific hybridization with a particular nucleic acid. Such oligonucleotides which specifically hybridize to a portion of the sense strand of a gene are commonly described as "antisense." In the context of the invention, "hybridization" means hydrogen bonding, which may be Watson-Crick, Hoogsteen or reversed Hoogsteen hydrogen bonding, between complementary nucleotides. For example, adenine and thymine are complementary nucleobases which pair through the formation of hydrogen bonds. "Complementary," as used herein, refers to the capacity for precise pairing between two nucleotides. For example, if a nucleotide at a certain position of an oligonucleotide is capable of hydrogen bonding with a nucleotide at the same position of a DNA or RNA molecule, then the oligonucleotide and the DNA or RNA are considered to be complementary to each other at that position. The oligonucleotide and the DNA or RNA are complementary to each other when a sufficient number of corresponding positions in each molecule are occupied by nucleotides which can hydrogen bond with each other. Thus, "specifically hybridizable" and "complementary" are terms which are used to indicate a sufficient degree of complementarity or precise pairing such that stable and specific binding occurs between the oligonucleotide and the DNA or RNA target. An oligonucleotide is specifically hybridizable to its target sequence due to the formation of base pairs between specific partner nucleobases in the interior of a nucleic acid duplex. Among the naturally occurring nucleobases, guanine (G) binds to cytosine (C), and adenine (A) binds to thymine (T) or uracil (U). In addition to the equivalency of U (RNA) and T (DNA) as partners for A, other naturally occurring nucleobase equivalents are known, including 5-methylcytosine, 5-hydroxymethylcytosine (HMC), glycosyl HMC and gentiobiosyl HMC (C equivalents), and 5-hydroxymethyluracil (U equivalent). Furthermore, synthetic

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nucleobases which retain partner specificity are known in the art and include, for example, 7-deaza-Guanine, which retains partner specificity for C. Thus, an oligonucleotide's capacity to specifically hybridize with its target sequence will not be altered by any chemical modification to a nucleobase in the nucleotide sequence of the oligonucleotide which does not significantly effect its specificity for the partner nucleobase in the target oligonucleotide. It is understood in the art that an oligonucleotide need not be 100% complementary to its target DNA sequence to be specifically hybridizable. An oligonucleotide is specifically hybridizable when there is a sufficient degree of complementarity to avoid non-specific binding of the oligonucleotide to non-target sequences under conditions in which specific binding is desired, i.e., under physiological conditions in the case of *in vivo* assays or therapeutic treatment, or in the case of *in vitro* assays, under conditions in which the assays are performed. The nucleotide sequences of the oligonucleotides of the invention are given in Example 1 and also in the Sequence Listing. Citations for target H-ras sequences are also presented in Example 1.

Antisense oligonucleotides are commonly used as research reagents, diagnostic aids, and therapeutic agents. For example, antisense oligonucleotides, which are able to inhibit gene expression with exquisite specificity, are often used by those of ordinary skill to elucidate the function of particular genes, for example to distinguish between the functions of various members of a biological pathway. This specific inhibitory effect has, therefore, been harnessed by those skilled in the art for research uses. The specificity and sensitivity of oligonucleotides is also harnessed by those of skill in the art for therapeutic uses.

**A. Modified Linkages:** Specific examples of some preferred modified oligonucleotides envisioned for this

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invention include those containing phosphorothioates, phosphotriesters, methyl phosphonates, short chain alkyl or cycloalkyl intersugar linkages or short chain heteroatomic or heterocyclic intersugar linkages. Most preferred are  
5 oligonucleotides with phosphorothioates and those with  $\text{CH}_2\text{-NH-O-CH}_2$ ,  $\text{CH}_2\text{-N(CH}_3\text{)-O-CH}_2$  [known as a methylene(methylimino) or MMI backbone],  $\text{CH}_2\text{-O-N(CH}_3\text{)-CH}_2$ ,  $\text{CH}_2\text{-N(CH}_3\text{)-N(CH}_3\text{)-CH}_2$  and  $\text{O-N(CH}_3\text{)-CH}_2\text{-CH}_2$  backbones, wherein the native phosphodiester backbone is represented as  $\text{O-P-O-CH}_2$ . Also preferred are  
10 oligonucleotides having morpholino backbone structures (Summerton and Weller, U.S. Patent No. 5,034,506). Further preferred are oligonucleotides with  $\text{NR-C(*)-CH}_2\text{-CH}_2$ ,  $\text{CH}_2\text{-NR-C(*)-CH}_2$ ,  $\text{CH}_2\text{-CH}_2\text{-NR-C(*)}$ ,  $\text{C(*)-NR-CH}_2\text{-CH}_2$  and  $\text{CH}_2\text{-C(*)-NR-CH}_2$  backbones, wherein "\*" represents O or S (known as amide  
15 backbones; DeMesmaeker et al., WO 92/20823, published November 26, 1992). In other preferred embodiments, such as the peptide nucleic acid (PNA) backbone, the phosphodiester backbone of the oligonucleotide is replaced with a polyamide backbone, the nucleobases being bound directly or indirectly  
20 to the aza nitrogen atoms of the polyamide backbone (Nielsen et al., *Science*, 1991, 254:1497; U.S. Patent No. 5,539,082).

**B. Modified Nucleobases:** The oligonucleotides of the invention may additionally or alternatively include nucleobase modifications or substitutions. As used herein, "unmodified"  
25 or "natural" nucleobases include adenine (A), guanine (G), thymine (T), cytosine (C) and uracil (U). Modified nucleobases include nucleobases found only infrequently or transiently in natural nucleic acids, e.g., hypoxanthine, 6-methyladenine, 5-methylcytosine, 5-hydroxymethylcytosine  
30 (HMC), glycosyl HMC and gentiobiosyl HMC, as well synthetic nucleobases, e.g., 2-aminoadenine, 2-thiouracil, 2-thiothymine, 5-bromouracil, 5-hydroxymethyluracil, 8-azaguanine, 7-deazaguanine,  $\text{N}^6$ (6-aminohexyl)adenine and 2,6-diaminopurine (Kornberg, A., *DNA Replication*, W.H. Freeman &

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Co., San Francisco, 1980, pages 75-77; Gebeyehu, G., et al., *Nucleic Acids Res.*, 1987, 15, 4513).

**C. Sugar Modifications:** The oligonucleotides of the invention may additionally or alternatively comprise  
5 substitutions of the sugar portion of the individual nucleotides. For example, oligonucleotides may also have sugar mimetics such as cyclobutyls in place of the pentofuranosyl group. Other preferred modified oligonucleotides may contain one or more substituted sugar  
10 moieties comprising one of the following at the 2' position: OH, SH, SCH<sub>3</sub>, F, OCN, OCH<sub>3</sub>OCH<sub>3</sub>, OCH<sub>3</sub>O(CH<sub>2</sub>)<sub>n</sub>CH<sub>3</sub>, O(CH<sub>2</sub>)<sub>n</sub>NH<sub>2</sub> or O(CH<sub>2</sub>)<sub>n</sub>CH<sub>3</sub> where n is from 1 to about 10; C<sub>1</sub> to C<sub>10</sub> lower alkyl, alkoxyalkoxy, substituted lower alkyl, alkaryl or aralkyl; Cl; Br; CN; CF<sub>3</sub>; OCF<sub>3</sub>; O-, S-, or N-alkyl; O-, S-, or N-alkenyl;  
15 SOCH<sub>3</sub>; SO<sub>2</sub>CH<sub>3</sub>; ONO<sub>2</sub>; NO<sub>2</sub>; N<sub>3</sub>; NH<sub>2</sub>; heterocycloalkyl; heterocycloalkaryl; aminoalkylamino; polyalkylamino; substituted silyl; an RNA cleaving group; a reporter group; an intercalator; a group for improving the pharmacokinetic properties of an oligonucleotide; or a group for improving the  
20 pharmacodynamic properties of an oligonucleotide and other substituents having similar properties. A preferred modification includes 2'-methoxyethoxy [2'-O-CH<sub>2</sub>CH<sub>2</sub>OCH<sub>3</sub>, also known as 2'-O-(2-methoxyethyl)] (Martin et al., *Helv. Chim. Acta*, 1995, 78:486). Other preferred modifications include  
25 2'-methoxy- (2'-O-CH<sub>3</sub>), 2'-propoxy- (2'-OCH<sub>2</sub>CH<sub>2</sub>CH<sub>3</sub>) and 2'-fluoro- (2'-F).

**D. Other Modifications:** Similar modifications may also be made at other positions on the oligonucleotide, particularly the 3' position of the sugar on the 3' terminal  
30 nucleotide and the 5' position of 5' terminal nucleotide. The 5' and 3' termini of an oligonucleotide may also be modified to serve as points of chemical conjugation of, e.g., lipophilic moieties (see immediately subsequent paragraph), intercalating agents (Kuyavin et al., WO 96/32496, published



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October 17, 1996; Nguyen et al., U.S. Patent No. 4,835,263, issued May 30, 1989) or hydroxyalkyl groups (Helene et al., WO 96/34008, published October 31, 1996).

Other positions within an oligonucleotide of the invention can be used to chemically link thereto one or more effector groups to form an oligonucleotide conjugate. An "effector group" is a chemical moiety that is capable of carrying out a particular chemical or biological function. Examples of such effector groups include, but are not limited to, an RNA cleaving group, a reporter group, an intercalator, a group for improving the pharmacokinetic properties of an oligonucleotide, or a group for improving the pharmacodynamic properties of an oligonucleotide and other substituents having similar properties. A variety of chemical linkers may be used to conjugate an effector group to an oligonucleotide of the invention. As an example, U.S. Patent No. 5,578,718 to Cook et al. discloses methods of attaching an alkylthio linker, which may be further derivatized to include additional groups, to ribofuranosyl positions, nucleosidic base positions, or on internucleoside linkages. Additional methods of conjugating oligonucleotides to various effector groups are known in the art; see, e.g., *Protocols for Oligonucleotide Conjugates (Methods in Molecular Biology, Volume 26)* Agrawal, S., ed., Humana Press, Totowa, NJ, 1994.

Another preferred additional or alternative modification of the oligonucleotides of the invention involves chemically linking to the oligonucleotide one or more lipophilic moieties which enhance the cellular uptake of the oligonucleotide. Such lipophilic moieties may be linked to an oligonucleotide at several different positions on the oligonucleotide. Some preferred positions include the 3' position of the sugar of the 3' terminal nucleotide, the 5' position of the sugar of the 5' terminal nucleotide, and the 2' position of the sugar of any nucleotide. The N<sup>c</sup> position

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of a purine nucleobase may also be utilized to link a lipophilic moiety to an oligonucleotide of the invention (Gebeyehu, G., et al., *Nucleic Acids Res.*, 1987, 15:4513). Such lipophilic moieties include but are not limited to a cholesteryl moiety (Letsinger et al., *Proc. Natl. Acad. Sci. U.S.A.*, 1989, 86:6553), cholic acid (Manoharan et al., *Bioorg. Med. Chem. Lett.*, 1994, 4:1053), a thioether, e.g., hexyl-S-tritylthiol (Manoharan et al., *Ann. N.Y. Acad. Sci.*, 1992, 660:306; Manoharan et al., *Bioorg. Med. Chem. Lett.*, 1993, 3:2765), a thiocholesterol (Oberhauser et al., *Nucl. Acids Res.*, 1992, 20:533), an aliphatic chain, e.g., dodecandiol or undecyl residues (Saison-Behmoaras et al., *EMBO J.*, 1991, 10:111; Kabanov et al., *FEBS Lett.*, 1990, 259:327; Svinarchuk et al., *Biochimie*, 1993, 75:49), a phospholipid, e.g., dihexadecyl-rac-glycerol or triethylammonium 1,2-di-O-hexadecyl-rac-glycero-3-H-phosphonate (Manoharan et al., *Tetrahedron Lett.*, 1995, 36:3651; Shea et al., *Nucl. Acids Res.*, 1990, 18:3777), a polyamine or a polyethylene glycol chain (Manoharan et al., *Nucleosides & Nucleotides*, 1995, 14:969), or adamantane acetic acid (Manoharan et al., *Tetrahedron Lett.*, 1995, 36:3651), a palmityl moiety (Mishra et al., *Biochim. Biophys. Acta*, 1995, 1264:229), or an octadecylamine or hexylamino-carbonyl-oxycholesterol moiety (Crooke et al., *J. Pharmacol. Exp. Ther.*, 1996, 277:923). Oligonucleotides comprising lipophilic moieties, and methods for preparing such oligonucleotides, are disclosed in co-owned U.S. Patents Nos. 5,138,045, 5,218,105 and 5,459,255.

The present invention also includes oligonucleotides that are substantially chirally pure with regard to particular positions within the oligonucleotides. Examples of substantially chirally pure oligonucleotides include, but are not limited to, those having phosphorothioate linkages that are at least 75% Sp or Rp (see co-owned U.S. Patent No. 5,587,361 to Cook et al.) and those having substantially

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chirally pure (Sp or Rp) alkylphosphonate, phosphoamidate or phosphotriester linkages (see co-owned U.S. Patents Nos. 5,212,295 and 5,521,302).

**E. Chimeric Oligonucleotides:** The present invention also includes oligonucleotides which are chimeric oligonucleotides. "Chimeric" oligonucleotides or "chimeras," in the context of this invention, are oligonucleotides which contain two or more chemically distinct regions, each made up of at least one nucleotide. These oligonucleotides typically contain at least one region wherein the oligonucleotide is modified so as to confer upon the oligonucleotide increased resistance to nuclease degradation, increased cellular uptake, and/or increased binding affinity for the target nucleic acid. An additional region of the oligonucleotide may serve as a substrate for enzymes capable of cleaving RNA:DNA or RNA:RNA hybrids. By way of example, RNase H is a cellular endonuclease which cleaves the RNA strand of an RNA:DNA duplex. Activation of RNase H, therefore, results in cleavage of the RNA target, thereby greatly enhancing the efficiency of antisense inhibition of gene expression. Cleavage of the RNA target can be routinely detected by gel electrophoresis and, if necessary, associated nucleic acid hybridization techniques known in the art. By way of example, such "chimeras" may be "gapmers," i.e., oligonucleotides in which a central portion (the "gap") of the oligonucleotide serves as a substrate for, e.g., RNase H, and the 5' and 3' portions (the "wings") are modified in such a fashion so as to have greater affinity for the target RNA molecule but are unable to support nuclease activity (e.g., 2'-fluoro- or 2'-methoxyethoxy- substituted). Other chimeras include "wingmers," that is, oligonucleotides in which the 5' portion of the oligonucleotide serves as a substrate for, e.g., RNase H, whereas the 3' portion is modified in such a fashion so as to have greater affinity for the target RNA molecule but is

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unable to support nuclease activity (e.g., 2'-fluoro- or 2'-methoxyethoxy- substituted), or vice-versa.

**F. Synthesis:** The oligonucleotides used in accordance with this invention may be conveniently and  
5 routinely made through the well-known technique of solid phase synthesis. Equipment for such synthesis is sold by several vendors including, for example, Applied Biosystems (Foster City, CA). Any other means for such synthesis known in the art may additionally or alternatively be employed. It is also  
10 known to use similar techniques to prepare other oligonucleotides such as the phosphorothioates and alkylated derivatives.

**1. Teachings:** regarding the synthesis of particular modified oligonucleotides may be found in the  
15 following U.S. patents or pending patent applications, each of which is commonly assigned with this application and is hereby incorporated by reference: U.S. Patents Nos. 5,138,045 and 5,218,105, drawn to polyamine conjugated oligonucleotides; U.S. Patent No. 5,212,295, drawn to monomers for the  
20 preparation of oligonucleotides having chiral phosphorus linkages; U.S. Patents Nos. 5,378,825 and 5,541,307, drawn to oligonucleotides having modified backbones; U.S. Patent No. 5,386,023, drawn to backbone modified oligonucleotides and the preparation thereof through reductive coupling; U.S. Patent  
25 No. 5,457,191, drawn to modified nucleobases based on the 3-deazapurine ring system and methods of synthesis thereof; U.S. Patent No. 5,459,255, drawn to modified nucleobases based on N-2 substituted purines; U.S. Patent No. 5,521,302, drawn to processes for preparing oligonucleotides having chiral  
30 phosphorus linkages; U.S. Patent No. 5,539,082, drawn to peptide nucleic acids; U.S. Patent No. 5,554,746, drawn to oligonucleotides having b-lactam backbones; U.S. Patent No. 5,571,902, drawn to methods and materials for the synthesis of oligonucleotides; U.S. Patent No. 5,578,718, drawn to

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nucleosides having alkylthio groups, wherein such groups may be used as linkers to other moieties attached at any of a variety of positions of the nucleoside; U.S. Patents Nos. 5,587,361 and 5,599,797, drawn to oligonucleotides having phosphorothioate linkages of high chiral purity; U.S. Patent No. 5,506,351, drawn to processes for the preparation of 2'-O-alkyl guanosine and related compounds, including 2,6-diaminopurine compounds; U.S. Patent No. 5,587,469, drawn to oligonucleotides having N-2 substituted purines; U.S. Patent No. 5,587,470, drawn to oligonucleotides having 3-deazapurines; U.S. Patents Nos. 5,223,168, issued June 29, 1993, and 5,608,046, both drawn to conjugated 4'-desmethyl nucleoside analogs; U.S. Patent Nos. 5,602,240, and 5,610,289, drawn to backbone modified oligonucleotide analogs; and U.S. patent application Serial No. 08/383,666, filed February 3, 1995, and U.S. Patent No. 5,459,255, drawn to, *inter alia*, methods of synthesizing 2'-fluoro-oligonucleotides. In 2'-methoxyethoxy-modified oligonucleotides, 5-methyl-2'-methoxyethoxy-cytosine residues are used and prepared as described in pending application Serial No. 08/731,199, filed October 4, 1996. Specific methods for preparing MMI linkages are taught in United States Patent Nos. 5,378,825 (issued January 3, 1995), 5,386,023 (issued January 31, 1995), 5,489,243 (issued on February 6, 1996), 5,541,307 (issued on July 30, 1996), 5,618,704 (issued April 8, 1997) and 5,623,070 (issued April 22, 1997). MMI is an abbreviation for methylene(methylimino) that in turn is a shorten version of the more complex chemical nomenclature "3'-de(oxyphosphinico)-3'[methylene(methylimino)]." Irrespective of chemical nomenclature, the linkages are as described in these patents. The linkages of these patents have also been described in various scientific publications by the inventors and their co-authors including Bhat et al. (*J. Org. Chem.* 61:8186, 1996, and references cited therein).

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2. **Bioequivalents:** The compounds of the invention encompass any pharmaceutically acceptable salts, esters, or salts of such esters, or any other compound which, upon administration to an animal including a human, is capable of providing (directly or indirectly) the biologically active metabolite or residue thereof. Accordingly, for example, the disclosure is also drawn to "prodrugs" and "pharmaceutically acceptable salts" of the oligonucleotides of the invention, pharmaceutically acceptable salts of such prodrugs, and other bioequivalents.

**A. Oligonucleotide Prodrugs:**

The oligonucleotides of the invention may additionally or alternatively be prepared to be delivered in a "prodrug" form. The term "prodrug" indicates a therapeutic agent that is prepared in an inactive form that is converted to an active form (i.e., drug) within the body or cells thereof by the action of endogenous enzymes or other chemicals and/or conditions. In particular, prodrug versions of the oligonucleotides of the invention are prepared as SATE [(S-acetyl-2-thioethyl) phosphate] derivatives according to the methods disclosed in WO 93/24510 to Gosselin *et al.*, published December 9, 1993.

**B. Pharmaceutically Acceptable Salts:** The term "pharmaceutically acceptable salts" refers to physiologically and pharmaceutically acceptable salts of the oligonucleotides of the invention: i.e., salts that retain the desired biological activity of the parent compound and do not impart undesired toxicological effects thereto.

Pharmaceutically acceptable base addition salts are formed with metals or amines, such as alkali and alkaline earth metals or organic amines. Examples of metals used as cations are sodium, potassium, magnesium, calcium, and the like. Examples of suitable amines are N,N'-dibenzylethylenediamine, chloroprocaine, choline,

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diethanolamine, dicyclohexylamine, ethylenediamine, N-methylglucamine, and procaine (see, for example, Berge et al., "Pharmaceutical Salts," *J. of Pharma Sci.*, 1977, 66:1). The base addition salts of said acidic compounds are prepared

5 by contacting the free acid form with a sufficient amount of the desired base to produce the salt in the conventional manner. The free acid form may be regenerated by contacting the salt form with an acid and isolating the free acid in the conventional manner. The free acid forms differ from their

10 respective salt forms somewhat in certain physical properties such as solubility in polar solvents, but otherwise the salts are equivalent to their respective free acid for purposes of the present invention. As used herein, a "pharmaceutical addition salt" includes a pharmaceutically acceptable salt of

15 an acid form of one of the components of the compositions of the invention. These include organic or inorganic acid salts of the amines. Preferred acid salts are the hydrochlorides, acetates, salicylates, nitrates and phosphates. Other suitable pharmaceutically acceptable salts are well known to

20 those skilled in the art and include basic salts of a variety of inorganic and organic acids, such as, for example, with inorganic acids, such as for example hydrochloric acid, hydrobromic acid, sulfuric acid or phosphoric acid; with organic carboxylic, sulfonic, sulfo or phospho acids or

25 N-substituted sulfamic acids, for example acetic acid, propionic acid, glycolic acid, succinic acid, maleic acid, hydroxymaleic acid, methylmaleic acid, fumaric acid, malic acid, tartaric acid, lactic acid, oxalic acid, gluconic acid, glucaric acid, glucuronic acid, citric acid, benzoic acid,

30 cinnamic acid, mandelic acid, salicylic acid, 4-aminosalicylic acid, 2-phenoxybenzoic acid, 2-acetoxybenzoic acid, embonic acid, nicotinic acid or isonicotinic acid; and with amino acids, such as the 20 alpha-amino acids involved in the synthesis of proteins in nature, for example glutamic acid or

35 aspartic acid, and also with phenylacetic acid,

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methanesulfonic acid, ethanesulfonic acid, 2-hydroxyethanesulfonic acid, ethane-1,2-disulfonic acid, benzenesulfonic acid, 4-methylbenzenesulfonic acid, naphthalene-2-sulfonic acid, naphthalene-1,5-disulfonic acid, 5 2- or 3-phosphoglycerate, glucose-6-phosphate, N-cyclohexylsulfamic acid (with the formation of cyclamates), or with other acid organic compounds, such as ascorbic acid. Pharmaceutically acceptable salts of compounds may also be prepared with a pharmaceutically acceptable cation including, 10 for example, alkaline, alkaline earth, ammonium and quaternary ammonium cations. Carbonates or hydrogen carbonates are also possible.

For oligonucleotides, preferred examples of pharmaceutically acceptable salts include but are not limited 15 to (a) salts formed with cations such as sodium, potassium, ammonium, magnesium, calcium, polyamines such as spermine and spermidine, etc.; (b) acid addition salts formed with inorganic acids, for example hydrochloric acid, hydrobromic acid, sulfuric acid, phosphoric acid, nitric acid and the 20 like; (c) salts formed with organic acids such as, for example, acetic acid, oxalic acid, tartaric acid, succinic acid, maleic acid, fumaric acid, gluconic acid, citric acid, malic acid, ascorbic acid, benzoic acid, tannic acid, palmitic acid, alginic acid, polyglutamic acid, 25 naphthalenesulfonic acid, methanesulfonic acid, p-toluenesulfonic acid, naphthalenedisulfonic acid, polygalacturonic acid, and the like; and (d) salts formed from elemental anions such as chlorine, bromine, and iodine.

**3. Sterically Stabilized Liposomes:** In 30 compositions of the invention, one or more antisense oligonucleotides and/or therapeutic agents are entrapped within liposomes. Liposomes are microscopic spheres having an aqueous core surrounded by one or more outer layer(s) made up of lipids arranged in a bilayer configuration (see,



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generally, Chonn *et al.*, *Current Op. Biotech.* 6:698, 1995). The therapeutic potential of liposomes as drug delivery agents was recognized nearly thirty years ago (Sessa *et al.*, *J. Lipid Res.* 9:310, 1968). Liposomes include "sterically stabilized liposome," a term which, as used herein, refers to a liposome comprising one or more specialized lipids that, when incorporated into liposomes, result in enhanced circulation lifetimes relative to liposomes lacking such specialized lipids. Examples of sterically stabilized liposomes are those in which part of the vesicle-forming lipid portion of the liposome (A) comprises one or more glycolipids, such as monosialoganglioside  $G_{M1}$ , or (B) is derivatized with one or more hydrophilic polymers, such as a polyethylene glycol (PEG) moiety. While not wishing to be bound by any particular theory, it is thought in the art that, at least for sterically stabilized liposomes containing gangliosides, sphingomyelin, or PEG-derivatized lipids, the enhanced circulation half-life of these sterically stabilized liposomes derives from a reduced uptake into cells of the reticuloendothelial system (RES) (Allen *et al.*, *FEBS Letters* 223:42, 1987; Wu *et al.*, *Cancer Research* 53:3765, 1993).

**A. Glycolipid-comprising liposomes:** Various liposomes comprising one or more glycolipids are known in the art. Papahadjopoulos *et al.* (*Ann. N.Y. Acad. Sci.*, 507:64, 1987) reported the ability of monosialoganglioside  $G_{M1}$ , galactocerebroside sulfate and phosphatidylinositol to improve blood half-lives of liposomes. These findings were expounded upon by Gabizon *et al.* (*Proc. Natl. Acad. Sci. U.S.A.* 85:6949, 1988). U.S. Patent No. 4,837,028 and WO 88/04924, both to Allen *et al.*, disclose liposomes comprising (1) sphingomyelin and (2) the ganglioside  $G_{M1}$  or a galactocerebroside sulfate ester. U.S. Patent No. 5,543,152 (Webb *et al.*) discloses liposomes comprising sphingomyelin. Liposomes comprising 1,2-

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sn-dimyristoylphosphatidylcholine are disclosed in WO 97/13499 (Lim et al.).

**B. Liposomes derivatized with hydrophilic polymers:**

Many liposomes comprising lipids derivatized with one or more hydrophilic polymers, and methods of preparation thereof, are known in the art. Sunamoto et al. (*Bull. Chem. Soc. Jpn.* 53:2778, 1980) described liposomes comprising a nonionic detergent, 2C<sub>12</sub>15G, that contains a PEG moiety. Illum et al. (*FEBS Letters* 167:79, 1984) noted that hydrophilic coating of polystyrene particles with polymeric glycols results in significantly enhanced blood half-lives. Synthetic phospholipids modified by the attachment of carboxylic groups of polyalkylene glycols (e.g., PEG) are described by Sears (U.S. Patent Nos. 4,426,330 and 4,534,899). Klibanov et al. (*FEBS Letts.* 268:235, 1990) described experiments demonstrating that liposomes comprising phosphatidylethanolamine (PE) derivatized with PEG or PEG stearate have significant increases in blood circulation half-lives. Blume et al. (*Biochimica et Biophysica Acta* 1029:91, 1990) extended such observations to other PEG-derivatized phospholipids, e.g., DSPE-PEG, formed from the combination of distearoylphosphatidylethanolamine (DSPE) and PEG. Liposomes having covalently bound PEG moieties on their external surface are described in European Patent No. 0 445 131 B1 and WO 90/04384 to Fisher. Liposome compositions containing 1-20 mole percent of PE derivatized with PEG, and methods of use thereof, are described by Woodle et al. (U.S. Patent Nos. 5,013,556 and 5,356,633) and Martin et al. (U.S. Patent No. 5,213,804 and European Patent No. EP 0 496 813 B1). Liposomes comprising a number of other lipid-polymer conjugates are disclosed in WO 91/05545 and U.S. Patent No. 5,225,212 (both to Martin et al.) and in WO 94/20073 (Zalipsky et al.) Liposomes comprising PEG-modified ceramide lipids are described in WO 96/10391 (Choi et al.). U.S. Patent Nos.

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5,540,935 (Miyazaki et al.) and 5,556,948 (Tagawa et al.) describe PEG-containing liposomes that can be further derivatized with functional moieties on their surfaces.

**C. Liposomes comprising nucleic acids:** A limited number of liposomes comprising nucleic acids are known in the art. WO 96/40062 to Thierry et al. discloses methods for encapsulating high molecular weight nucleic acids in liposomes. U.S. Patent No. 5,264,221 to Tagawa et al. discloses protein-bonded liposomes and asserts that the contents of such liposomes may include an antisense RNA. U.S. Patent No. 5,665,710 to Rahman et al. describes certain methods of encapsulating oligodeoxynucleotides in liposomes. WO 97/04787 to Love et al. discloses liposomes comprising antisense oligonucleotides targeted to the raf gene.

**4. Chemotherapeutic Agents:** Certain embodiments of the invention provide for liposomes containing (a) one or more antisense oligonucleotides targeted to a nucleic acid encoding a ras protein and (b) one or more chemotherapeutic agents which do not function by an antisense mechanism. In a related embodiment, such chemotherapeutic agents are co-administered with one or more of the liposomal oligonucleotide compositions of the invention but are separately encapsulated in distinct liposomes or are administered by a non-liposomal delivery mechanism. As used herein, a "chemotherapeutic agent" is an anticancer agent that functions via a conventional (i.e., non-antisense) mode of action. Examples of such chemotherapeutic agents include, but are not limited to, daunorubicin, dactinomycin, doxorubicin, bleomycin, mitomycin, nitrogen mustard, chlorambucil, melphalan, cyclophosphamide, 6-mercaptapurine, 6-thioguanine, cytarabine (CA), 5-fluorouracil (5-FU), floxuridine (5-FUdR), methotrexate (MTX), colchicine, vincristine, vinblastine, etoposide, teniposide, cisplatin and diethylstilbestrol (DES). See, generally, *The Merck Manual of Diagnosis and Therapy*, 15th Ed., Berkow et

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al., eds., 1987, Rahay, N.J., pages 1206-1228). When used with the liposomal oligonucleotide compositions of the invention, such chemotherapeutic agents may be used individually, sequentially (e.g., 5-FU for a period of time  
5 followed by MTX), or in combination with one or more other such chemotherapeutic agents (e.g., 5-FU and MTX).

In a related embodiment, liposomes containing (a) one or more antisense oligonucleotides targeted to a first nucleic acid encoding a ras sequence and (b) one or more additional  
10 antisense oligonucleotides targeted to a second nucleic acid encoding a cancer associated gene. By the term "cancer associated gene" is intended any cellular or viral gene the expression of which disrupts regulation of the cell cycle, negatively effects contact inhibition of growth, leads to  
15 cellular hyperproliferation, promotes pre-metastatic or metastatic events and/or otherwise leads to cellular hyperproliferation, tumor formation and the growth and spread of cancers, regardless of mechanism of action. In a related embodiment, such additional antisense oligonucleotides  
20 targeted to a second cancer-associated gene are co-administered with one or more of the liposomal oligonucleotide compositions of the invention but are separately encapsulated in distinct liposomes or are administered by a non-liposomal delivery mechanism. Such antisense oligonucleotides targeted  
25 to a second cancer associated gene include, but are not limited to, those directed to the following targets as disclosed in the indicated co-owned U.S. Patents, pending applications or published PCT applications, which are hereby incorporated by reference: raf (WO 96/39415, WO 95/32987 and  
30 U.S. Patent Nos. 5,563,255, issued October 8, 1996, and 5,656,612, issued August 12, 1997), the p120 nucleolar antigen (WO 93/17125 and U.S. Patent No. 5,656,743, issued August 12, 1997), protein kinase C (WO 95/02069, WO 95/03833 and WO 93/19203), multidrug resistance-associated protein (WO

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95/10938 and U.S. Patent No. 5,510,239, issued March 23, 1996), subunits of transcription factor AP-1 (co-pending application U.S. Serial No. 08/837,201, filed April 14, 1997), Jun kinases (co-pending application U.S. Serial No. 5 08/910,629, filed August 13, 1997), and MDR-1 (multidrug resistance glycoprotein; co-pending application U.S. Serial No. 08/731,199, filed September 30, 1997).

#### 5. Administration of Pharmaceutical

**Compositions:** The formulation of pharmaceutical compositions comprising the liposomal oligonucleotide compositions of the invention and their subsequent administration is believed to be within the skill of those in the art. In general, for therapeutics, a patient in need of such therapy is administered a liposomal oligonucleotide composition in 15 accordance with the invention, commonly in a pharmaceutically acceptable carrier, in doses ranging from 0.01 µg to 100 g per kg of body weight depending on the age of the patient and the severity of the disorder or disease state being treated. Dosing is dependent on severity and responsiveness of the 20 disease state to be treated, with the course of treatment lasting from several days to several months, or until a cure is effected or a diminution of the disease state is achieved. Optimal dosing schedules can be calculated from measurements of drug accumulation in the body of the patient. Persons of 25 ordinary skill can easily determine optimum dosages, dosing methodologies and repetition rates. Optimum dosages may vary depending on the relative potency of individual oligonucleotides, and can generally be estimated based on EC<sub>50</sub>s found to be effective in *in vitro* and *in vivo* animal models. 30 Further, the treatment regimen may last for a period of time which will vary depending upon the nature of the particular disease or disorder, its severity and the overall condition of the patient, and may extend from once daily to once every 20 years. Following treatment, the patient is monitored for

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changes in his/her condition and for alleviation of the symptoms of the disorder or disease state. The dosage of the oligonucleotide may either be increased in the event the patient does not respond significantly to current dosage  
5 levels, or the dose may be decreased if an alleviation of the symptoms of the disorder or disease state is observed, or if the disorder or disease state has been ablated.

An optimal dosing schedule is used to deliver a therapeutically effective amount of the oligonucleotide being  
10 administered via a particular mode of administration. The term "therapeutically effective amount," for the purposes of the invention, refers to the amount of oligonucleotide-containing pharmaceutical composition which is effective to achieve an intended purpose without undesirable side effects  
15 (such as toxicity, irritation or allergic response). Although individual needs may vary, determination of optimal ranges for effective amounts of pharmaceutical compositions is within the skill of the art. Human doses can be extrapolated from animal studies (Katocs et al., Chapter 27 In: Remington's  
20 *Pharmaceutical Sciences*, 18th Ed., Gennaro, ed., Mack Publishing Co., Easton, PA, 1990). Generally, the dosage required to provide an effective amount of a pharmaceutical composition, which can be adjusted by one skilled in the art, will vary depending on the age, health, physical condition,  
25 weight, type and extent of the disease or disorder of the recipient, frequency of treatment, the nature of concurrent therapy (if any) and the nature and scope of the desired effect(s) (Nies et al., Chapter 3 In: *Goodman & Gilman's The Pharmacological Basis of Therapeutics*, 9th Ed., Hardman et  
30 al., eds., McGraw-Hill, New York, NY, 1996).

In some cases it may be more effective to treat a patient with a liposomal oligonucleotide composition of the invention in conjunction with other, traditional therapeutic modalities in order to increase the efficacy of a treatment

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regimen. In the context of the invention, the term "treatment regimen" is meant to encompass therapeutic, palliative and prophylactic modalities. Following treatment, the patient is monitored for changes in his/her condition and for alleviation of the symptoms of the disorder or disease state. The dosage of the pharmaceutical composition may either be increased in the event the patient does not respond significantly to current dosage levels, or the dose may be decreased if an alleviation of the symptoms of the disorder or disease state is observed, or if the disorder or disease state has been ablated.

As used herein, the term "high risk individual" is meant to refer to an individual for whom it has been determined, via, e.g., individual or family history or genetic testing, that there is a significantly higher than normal probability of being susceptible to the onset or recurrence of a disease or disorder. As part of a treatment regimen for a high risk individual, the individual can be prophylactically treated to prevent the onset or recurrence of the disease or disorder. The term "prophylactically effective amount" is meant to refer to an amount of a pharmaceutical composition which produces an effect observed as the prevention of the onset or recurrence of a disease or disorder. Prophylactically effective amounts of a pharmaceutical composition are typically determined by the effect they have compared to the effect observed when a second pharmaceutical composition lacking the active agent is administered to a similarly situated individual.

The pharmaceutical compositions of the present invention may be administered in a number of ways depending upon whether local or systemic treatment is desired and upon the area to be treated. Typically, parenteral administration is employed. The term "parenteral delivery" refers to the administration of an oligonucleotide of the invention to an

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animal in a manner other than through the digestive canal. Parenteral administration includes intravenous (i.v.) drip, subcutaneous, intraperitoneal (i.p.) or intramuscular injection, or intrathecal or intraventricular administration.

5 Compositions for parenteral, intrathecal or intraventricular administration may include sterile aqueous solutions which may also contain buffers, diluents and other suitable additives. Means of preparing and administering parenteral pharmaceutical compositions are known in the art (see, e.g., Avis, Chapter  
10 84 *In: Remington's Pharmaceutical Sciences*, 18th Ed., Gennaro, ed., Mack Publishing Co., Easton, PA, 1990, pages 1545-1569). Parenteral means of delivery include, but are not limited to, the following illustrative examples.

(A) **Intravitreal injection**, for the direct  
15 delivery of drug to the vitreous humor of a mammalian eye, is described in U.S. Patent No. 5,591,720, the contents of which are hereby incorporated by reference. Means of preparing and administering ophthalmic preparations are known in the art (see, e.g., Mullins et al., Chapter 86 *In: Remington's*  
20 *Pharmaceutical Sciences*, 18th Ed., Gennaro, ed., Mack Publishing Co., Easton, PA, 1990, pages 1581-1595).

(B) **Intravenous administration** of antisense oligonucleotides to various non-human mammals has been described by Iversen (Chapter 26 *In: Antisense Research and*  
25 *Applications*, Crooke et al., eds., CRC Press, Boca Raton, FL, 1993, pages 461-469). Systemic delivery of oligonucleotides to non-human mammals via intraperitoneal means has also been described (Dean et al., *Proc. Natl. Acad. Sci. U.S.A.* 91:11766, 1994).

30 (C) **Intraluminal drug administration**, for the direct delivery of drug to an isolated portion of a tubular organ or tissue (e.g., such as an artery, vein, ureter or urethra), may be desired for the treatment of patients with diseases or conditions afflicting the lumen of such organs or



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tissues. To effect this mode of oligonucleotide administration, a catheter or cannula is surgically introduced by appropriate means. For example, for treatment of the left common carotid artery, a cannula is inserted therein via the external carotid artery. After isolation of a portion of the tubular organ or tissue for which treatment is sought, a composition comprising the oligonucleotides of the invention is infused through the cannula or catheter into the isolated segment. After incubation for from about 1 to about 120 minutes, during which the oligonucleotide is taken up by cells of the interior lumen of the vessel, the infusion cannula or catheter is removed and flow within the tubular organ or tissue is restored by removal of the ligatures which effected the isolation of a segment thereof (Morishita et al., *Proc. Natl. Acad. Sci. U.S.A.* 90:8474, 1993). Antisense oligonucleotides may also be combined with a biocompatible matrix, such as a hydrogel material, and applied directly to vascular tissue *in vivo* (Rosenberg et al., U.S. Patent No. 5,593,974, issued January 14, 1997).

(D) **Intraventricular drug administration**, for the direct delivery of drug to the brain of a patient, may be desired for the treatment of patients with diseases or conditions afflicting the brain. To effect this mode of oligonucleotide administration, a silicon catheter is surgically introduced into a ventricle of the brain of a human patient, and is connected to a subcutaneous infusion pump (Medtronic Inc., Minneapolis, MN) that has been surgically implanted in the abdominal region (Zimm et al., *Cancer Research* 44:1698, 1984; Shaw, *Cancer* 72(11 Suppl.):, 3416, 1993). The pump is used to inject the oligonucleotides and allows precise dosage adjustments and variation in dosage schedules with the aid of an external programming device. The reservoir capacity of the pump is 18-20 mL and infusion rates may range from 0.1 mL/h to 1 mL/h. Depending on the frequency

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of administration, ranging from daily to monthly, and the dose of drug to be administered, ranging from 0.01  $\mu$ g to 100 g per kg of body weight, the pump reservoir may be refilled at 3-10 week intervals. Refilling of the pump is accomplished by  
5 percutaneous puncture of the pump's self-sealing septum.

(E) **Intrathecal drug administration**, for the introduction of a drug into the spinal column of a patient may be desired for the treatment of patients with diseases of the central nervous system (CNS). To effect this route of  
10 oligonucleotide administration, a silicon catheter is surgically implanted into the L3-4 lumbar spinal interspace of a human patient, and is connected to a subcutaneous infusion pump which has been surgically implanted in the upper abdominal region (Luer and Hatton, *The Annals of*  
15 *Pharmacotherapy* 27:912, 1993; Ettinger et al. *Cancer*, 41:1270, 1978; Yaida et al., *Regul. Pept.* 59:193, 1985). The pump is used to inject the oligonucleotides and allows precise dosage adjustments and variations in dose schedules with the aid of an external programming device. The reservoir capacity of the  
20 pump is 18-20 mL, and infusion rates may vary from 0.1 mL/h to 1 mL/h. Depending on the frequency of drug administration, ranging from daily to monthly, and dosage of drug to be administered, ranging from 0.01  $\mu$ g to 100 g per kg of body weight, the pump reservoir may be refilled at 3-10 week  
25 intervals. Refilling of the pump is accomplished by a single percutaneous puncture to the self-sealing septum of the pump. The distribution, stability and pharmacokinetics of oligonucleotides within the CNS are followed according to known methods (Whitesell et al., *Proc. Natl. Acad. Sci. U.S.A.*  
30 90:4665, 1993).

To effect delivery of oligonucleotides to areas other than the brain or spinal column via this method, the silicon catheter is configured to connect the subcutaneous infusion pump to, e.g., the hepatic artery, for delivery to the liver

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(Kemeny et al., *Cancer* 71:1964, 1993). Infusion pumps may also be used to effect systemic delivery of oligonucleotides (Ewel et al., *Cancer Research* 52:3005, 1992; Rubenstein et al., *J. Surg. Oncol.* 62:194, 1996).

## 5 EXAMPLES

The following examples illustrate the invention and are not intended to limit the same. Those skilled in the art will recognize, or be able to ascertain through routine experimentation, numerous equivalents to the specific  
10 substances and procedures described herein. Such equivalents are considered to be within the scope of the present invention.

### EXAMPLE 1: Nucleic Acid Sequences

The oligonucleotides of this invention are designed  
15 to be complementary to, and thus hybridizable with, messenger RNA derived from a ras gene. Such hybridization, when accomplished, interferes with the normal roles of the messenger RNA to cause a loss of its function in the cell. The functions of messenger RNA to be interfered with include  
20 all vital functions such as translocation of the RNA to the site for protein translation, actual translation of protein from the RNA, splicing of the RNA to yield one or more mRNA species, and possibly even independent catalytic activity which may be engaged in by the RNA. The overall effect of  
25 such interference with the RNA function is to interfere with expression of the ras gene. Some oligonucleotides of this invention are designed to activate RNase H cleavage of the ras mRNA.

The protein products of the other mammalian ras  
30 genes, N-ras and K-ras, are identical to H-ras over the first 85 amino acids. However, the nucleic acid sequences of the three ras genes are not identical, and persons of ordinary

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skill in the art will be able to use this invention as a guide in preparing oligonucleotides specifically hybridizable with a particular ras gene. While one preferred embodiment of the invention relate to antisense oligonucleotides specifically  
5 hybridizable with codon 12 of the H-ras mRNA, the disclosure can be used by persons skilled in the art as a guide in preparing oligonucleotides specifically hybridizable with other point mutations of the H-ras gene, particularly the well defined point mutations at codon 12, codon 13 and codon 61 of  
10 H-ras, or point mutations within other ras genes.

The nucleotide sequence of wildtype (wt) H-ras, also known as Ha-ras, has been described by Capon et al. (*Nature* 302:33, 1983), Fasano et al. (*J. Mol. Appl. Genet.* 2:173, 1983), Reddy (*Science* 220:1061, 1983) and Honkawa et al. (*Mol.*  
15 *Cell. Biol.* 7:2933, 1987). Mutant (activated) H-ras sequences have been reported by Tabin et al. (*Nature* 300:143, 1982), Taparowsky et al. (*Nature* 300:762, 1982), Yuasa et al. (*Nature* 303:775, 1983), Sekiya et al. (*Proc. Natl. Acad. Sci. USA* 81:4771, 1984; *Jpn. J. Cancer Res.* 76:787, 1985), Kraus et al.  
20 (*Proc. Natl. Acad. Sci. USA* 81:5384, 1984), Stevens et al. (*Proc. Natl. Acad. Sci. USA* 85:3875), Deng et al. (*Cancer Res.* 47:3195, 1987), Santos et al. (*Proc. Natl. Acad. Sci. USA* 80:4679, 1983), Tanci et al. (*Nucleic Acids Res.* 20:1157, 1992) and Tadokoro et al. (*Oncogene* 4:499, 1989). The  
25 sequences of wildtype and mutant H-ras genes may also be found in the Genbank and EMBOL databases under Accession Nos. J00206, J00276, J00277, K00654, K00954, M30539, M19990, M17232, M25876, V00574, X01227 and X16438.

The nucleotide sequence of wildtype (wt) K-ras, also  
30 known as Ki-ras, has been described by McGrath et al. (*Nature* 304:501, 1983) and McCoy et al. (*Mol. Cell. Biol.* 4:1577, 1984). Mutant (activated) K-ras sequences have been reported by Shimizu et al. (*Nature* 304:497, 1983), Capon et al. (*Nature* 304:507, 1983), Nakano et al. (*Proc. Natl. Acad. Sci. U.S.A.*

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81:71, 1984), Taya et al. (EMBO J. 3:2943, 1984) and Nardeux et al. (Biochem. Biophys. Res. Commun. 146:395, 1987). The sequences of wildtype and mutant K-ras genes may also be found in Genbank under Accession Nos. K00652, K00653, K01519, 5 K01520, K01912, L00045, L00049, M17087, M26261, M38506 and M54968.

The nucleotide sequences of wildtype and mutant N-ras genes are known (Hall et al., Nucleic Acids Res. 13:5255, 1985; Taparowsky et al., Cell 34:581, 1983; Geis et al., 10 Biochem. Biophys. Res. Commun. 139:771, 1986; Brown et al., EMBO J. 3:1321, 1984). The sequences of wildtype and mutant N-ras genes may also be found in the Genbank and EMBOL databases under Accession Nos. K00082, L00043, M14307, X00645 and X02751.

15 Oligonucleotides targeted to ras genes are described in U.S. Patents Nos. 5,576,208; 5,582,972; 5,582,986; and 5,661,134, and pending application Serial No. 08/889,296, filed July 8, 1997, as well as WO 94/08003, WO 94/28720 and WO 92/22651 to Monia et al., all of which are assigned to the 20 same assignee as that of the present disclosure and which are hereby incorporated by reference.

The sequences and chemistries of oligonucleotides targeted to H-ras are detailed in Tables 1 through 7. The sequences and chemistries of oligonucleotides targeted to K- 25 ras are detailed in Table 8. Sequences and chemistries of oligonucleotides targeted to N-ras are detailed in Table 9.

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TABLE 1

## Phosphorothioate Antisense Oligodeoxynucleotides

## Targeted to H-ras

*Targeted to the H-ras translation initiation codon*

5	ISIS #	SEQUENCE (5'→3')	SEQ ID NO:
	2502	CTT-ATA-TTC-CGT-CAT-CGC-TC	1
	2503	TCC-GTC-ATC-GCT-CCT-CAG-GG	2
	2570	CCA-CAC-CGA-CGG-CGC-CC	3
	2571	CCC-ACA-CCG-ACG-GCG-CCC-A	4
10	2566	GCC-CAC-ACC-GAC-GGC-GCC-CAC	5
	2560	TGC-CCA-CAC-CGA-CGG-CGC-CCA-CC	6

*Targeted to mutant H-ras*

	ISIS #	TARGET	SEQUENCE (5'→3')	SEQ. ID NO:
	2502	AUG	CTTATATTCCGTCATCGCTC	1
15	2503	AUG	TCCGTCATCGCTCCTCAGGG	2
	6186	AUG	TATTCCGTCATCGCTCCTCA	7
	2563	CODON 12	CGACG	8
	2564	CODON 12	CCGACGG	9
	2565	CODON 12	ACCGACGGC	10
20	2567	CODON 12	CACCGACGGCG	11
	2568	CODON 12	ACACCGACGGCGC	12
	2569	CODON 12	CACACCGACGGCGCC	13
	3426	CODON 12	CCACACCGACGGCGCC	14
	3427	CODON 12	CACACCGACGGCGCCC	15
25	2570	CODON 12	CCACACCGACGGCGCCC	3
	3428	CODON 12	CCCACACCGACGGCGCCC	16
	3429	CODON 12	CCACACCGACGGCGCCCA	17
	2571	CODON 12	CCCACACCGACGGCGCCCA	4
	2566	CODON 12	GCCCACACCGACGGCGCCAC	5
30	2560	CODON 12	TGCCCACACCGACGGCGCCCACC	6
	2561	CODON 12	TTGCCCACACCGACGGCGCCCACCA	18

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2907                      CODON 12 (wt)      CCACACCGCCGGCGCCC                      19

TABLE 2

Chimeric Phosphorothioate Oligonucleotides Having  
2'-O-Methyl Ends (Bold) and Central Deoxy Gap

5 (Mutant Codon-12 Target)

# OF DEOXY

ISIS #	RESIDUES	SEQUENCE (5'→3')	SEQ ID NO:
4122	0	CCACACCGACGGCGCCC	3
3975	1	CCACACCGACGGCGCCC	3
10 3979	3	CCACACCGACGGCGCCC	3
4236	4	CCACACCGACGGCGCCC	3
4242	4	CCACACCGACGGCGCCC	3
3980	5	CCACACCGACGGCGCCC	3
3985	7	CCACACCGACGGCGCCC	3
15 3984	9	CCACACCGACGGCGCCC	3
2570	17	CCACACCGACGGCGCCC	3

TABLE 3

Shortened Phosphorothioate Chimeric Oligonucleotides  
Derived from ISIS 3980 Having 2'-O-Methyl Ends (Bold)

20 and Central Deoxy Gap (Mutant Codon-12 Target)

ISIS #	SEQUENCE (5'→3')	SEQ ID NO:
3980	CCACACCGACGGCGCCC	3
4230	CACACCGACGGCGCC	13
4276	ACACCGACGGCGC	12
25 4247	CACCGACGGCG	11
3985	CCACACCGACGGCGCCC	3

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4245	<b>CACACCGACGGCGCC</b>	13
4278	<b>ACACCGACGGCGC</b>	12
4229	<b>CACCGACGGCG</b>	11

TABLE 4

5                   Chimeric Phosphorothioate Oligonucleotides  
 Having 2'-O-Methyl Ends (Bold) and Central Deoxy Gap  
 (AUG Target)

	ISIS #	# OF DEOXY RESIDUES	SEQUENCE (5'→3')	SEQ ID NO:
10	2502	20	CTTATATTCCGTCATCGCTC	1
	4998	7	<b>CTTATATTCCGTCATCGCTC</b>	1
	2503	20	TCCGTCATCGCTCCTCAGGG	2
	5122	7	<b>TCCGTCATCGCTCCTCAGGG</b>	2

TABLE 5

15                   Chimeric Backbone (P=S/P=O) Oligonucleotides  
 Having 2'-O-Methyl Ends (Bold) and Central Deoxy Gap  
 (Backbone Linkages Indicated by "s" (P=S) or "o" (P=O))  
 (Mutant Codon-12 Target)

	ISIS #	# OF DEOXY RESIDUES	SEQUENCE (5'→3')	SEQ ID NO:
	2570	16	CsCsAsCsAsCsCsGsAsCsGsGsCsGsCsCsC	3
	4226	5	<b>CoCoAoCoAoCsCsGsAsCsGoGoCoGoCoCoC</b>	3
	4233	11	<b>CsCsAsCsAsCoCoGoAoCoGsGsCsGsCsCsC</b>	3
	4248	15	CsCsAsCsAsCsCsGsAoCsGsGsCsGsCsCsC	3
25	4546	14	CsCsAsCsAsCsCsGoAoCsGsGsCsGsCsCsC	3
	4551	13	CsCsAsCsAsCsCsGoAoCoGsGsCsGsCsCsC	3
	4593	12	CsCsAsCsAsCsCoGoAoCoGsGsCsGsCsCsC	3
	4606	11	CsCsAsCsAsCsCoGoAoCoGoGsCsGsCsCsC	3



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4241 6 CsCsAsCoAoCoCoGoAoCoGoGoCoGsCsCsC 3

TABLE 6

Phosphorothioate Antisense Oligodeoxynucleotides Targeted  
to a Hairpin Structure Corresponding to Residues

5	+18 to +64 of the Coding Sequence of Activated H-ras mRNA		
	ISIS #	SEQUENCE (5'→3')	SEQ ID NO:
	3270	CACCACCACC	20
	3271	GCGCCACCA	21
	3292	CGACGGCGCC	22
10	3291	CACACCGACG	23
	3283	UUGCCCACAC	24
	3284	CACUCUUGCC	25

TABLE 7

2'- Modified Analogs of ISIS 2503

15 (Positions with 2' Modifications are Emboldened)

MOE Analogs (positions with 2'-MOE are emboldened)

	ISIS #	Sequence (5'→3')	SEQ ID NO:
	13905	TCCGTCATCGCTCCTCAGGG	2
	13907	TCCGTCATCGCTCCTCAGGG	2
20	13909	TCCGTCATCGCTCCTCAGGG	2
	13911	TCCGTCATCGCTCCTCAGGG	2
	13917	TCCGTCATCGCTCCTCAGGG	2
	13919	TCCGTCATCGCTCCTCAGGG	2
	13920	TCCGTCATCGCTCCTCAGGG	2
25	13923	TCCGTCATCGCTCCTCAGGG	2
	13926	TCCGTCATCGCTCCTCAGGG	2
	13927	TCCGTCATCGCTCCTCAGGG	2

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**MMI Analogs (positions with 2'-MOE are emboldened)**

	ISIS #	Sequence (5'→3')	SEQ ID NO:
	14896	TCCGTCATCGCTCCTCAGGG	2
	14897	TC <sub>o</sub> CGTCATCGCTCCTCAG <sub>o</sub> GG	2
5	14898	TC <sub>s</sub> CGTCATCGCTCCTCAG <sub>s</sub> GG	2
	14899	TC <sub>o</sub> CG <sub>o</sub> TCATCGCTCCTC <sub>o</sub> A <sub>o</sub> GGG	2
	14900	TC <sub>s</sub> CG <sub>s</sub> TCATCGCTCCTC <sub>s</sub> AG <sub>s</sub> AG	2

"<sub>o</sub>" indicates a phosphodiester linkage between MMI dimers;

"<sub>s</sub>" indicates a phosphorothioate linkage between MMI dimers.

10 All unmarked linkages are phosphorothioates.

**TABLE 8****Phosphorothioate Antisense Oligonucleotides****Targeted to Human K-ras****Oligodeoxynucleotides**

	ISIS #	SEQUENCE (5'→3')	TARGET	SEQ ID NO:
15	6958	CTGCCTCCGCCGCGCGGCC	5' UTR/5'-cap	28
	6957	CAGTGCCTGCGCCGCGCTCG	5'-UTR	29
	6956	AGGCCTCTCTCCCGCACCTG	5'-UTR	30
	6953	TTCAGTCATTTTCAGCAGGC	AUG	31
20	6952	TTATATTCAGTCATTTTCAG	AUG	32
	6951	CAAGTTTATATTCAGTCATT	AUG	33
	6950	GCCTACGCCACCAGCTCCAAC	Codon 12 (wt)	34
	6949	CTACGCCACCAGCTCCA	Codon 12 (wt)	35
	7453	TACGCCAACAGCTCC	Codon 12 (G→T mutant)	36
25	6948	GTACTCCTCTTGACCTGCTGT	Codon 61 (wt)	37
	6947	CCTGTAGGAATCCTCTATTGT	Codon 38	38
	6946	GGTAATGCTAAAACAAATGC	3'-UTR	39
	6945	GGAATACTGGCACTTCGAGG	3'-UTR	40

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7679      TTTTCAGCAGGCCTCTCTCC      5'-UTR/AUG      41

**Chimeric oligonucleotides having 2'-O-methyl ends (bold)**

	ISIS #	SEQUENCE (5'→3')	SEQ ID NO:
	6957	CAGTGCCTGCGCCGCGCTCG	29
5	7683	<b>CAGTGCCTGCGCCGCGCTCG</b>	29
	7679	TTTTCAGCAGGCCTCTCTCC	41
	7680	<b>TTTTCAGCAGGCCTCTCTCC</b>	41

TABLE 9

**Phosphorothioate Oligodeoxynucleotides**

	ISIS #	Sequence (5'→3')	Target Region	SEQ ID NO:
	14677	CCGGGTCCTAGAAGCTGCAG	5' UTR	42
	14678	TAAATCAGTAAAAGAAACCG	5' UTR	43
	14679	GGACACAGTAACCAGGCGGC	5' UTR	44
15	14680	AACAGAAGCTACACCAAGGG	5' UTR	45
	14681	CAGACCCATCCATTCCCGTG	5' UTR	46
	14682	GCCAAGAAATCAGACCCATC	5' UTR	47
	14683	AGGGGGAAGATAAAACCGCC	5' UTR	48
	14684	CGCTTCCATTCTTTGCGCAT	5' UTR	49
20	14685	CCGCACCCAGACCCGCCCT	5' UTR	50
	14686	CAGCCCCCACCAAGGAGCGG	5' UTR	51
	14687	GTCATTTACACCAGCAAGA	AUG	52
	14688	CAGTCATTTACACCAGCAA	AUG	53
	14689	CTCAGTCATTTACACCAGC	AUG	54
25	14690	CGTGGGCTTGTTTGTATCA	Coding	55
	14691	CCATACAACCCTGAGTCCCA	3' UTR	56
	14692	CAGACAGCCAAGTGAGGAGG	3' UTR	57

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14693	CCAGGGCAGAAAAATAACAG	3' UTR	58
14694	TTTGTGCTGTGGAAGAACCC	3' UTR	59
14695	GCTATTAAATAACAATGCAC	3' UTR	60
14696	ACTGATCACAGCTATTAAAT	3' UTR	61

## 5 EXAMPLE 2: Oligonucleotide Synthesis

Substituted and unsubstituted deoxyoligonucleotides were synthesized on an automated DNA synthesizer (Applied Biosystems model 380B) using standard phosphoramidate chemistry with oxidation by iodine. For phosphorothioate oligonucleotides, the standard oxidation bottle was replaced by 0.2 M solution of 3H-1,2-benzodithiole-3-one 1,1-dioxide in acetonitrile for the stepwise thiation of the phosphite linkages. The thiation wait step was increased to 68 sec and was followed by the capping step. Synthesis of 2-(amino)adenine-substituted oligonucleotides was carried out in like manner, with the following exception: at positions at which a 2-(amino)adenine is desired, the standard phosphoramidite is replaced with a commercially available 2-aminodeoxyadenosine phosphoramidite (Chemgenes Corp., Waltham, MA). After cleavage from the CPG column and deblocking in concentrated ammonium hydroxide at 55°C (18 hr), the oligonucleotides were purified by precipitation twice out of 0.5 M NaCl solution with 2.5 volumes ethanol. Analytical gel electrophoresis was accomplished in 20% acrylamide, 8 M urea, 454 mM Tris-borate buffer, pH=7.0. Oligonucleotides were judged from polyacrylamide gel electrophoresis to be greater than 80% full-length material.

Oligoribonucleotides were synthesized using the automated synthesizer and 5'-dimethoxy-trityl 2'-tert-butyl dimethylsilyl 3'-O-phosphoramidites (American Bionetics, Hayward, CA). The protecting group on the exocyclic amines of A, C and G was phenoxyacetyl (Wu et al., *Nucl. Acids Res.* 17:3501, 1989). The standard synthesis cycle was modified by

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increasing the wait step after the pulse delivery of tetrazole to 900 seconds. Oligonucleotides were deprotected by overnight incubation at room temperature in methanolic ammonia. After drying in vacuo, the 2'-silyl group was removed by overnight incubation at room temperature in 1 M tetrabutylammoniumfluoride (Aldrich Chemical Co., Milwaukee, WI) in tetrahydrofuran. Oligonucleotides were purified using a C-18 Sep-Pak cartridge (Waters Corp., Milford, MA) followed by ethanol precipitation. Analytical denaturing polyacrylamide electrophoresis demonstrated the RNA oligonucleotides were greater than 90% full length material.

**EXAMPLE 3: Preparation of Sterically Stabilized Liposomes  
Comprising Antisense Oligonucleotides**

**A. Preparation of lipid film**

Lipid stock solutions were prepared at 20 mg/mL in chloroform. Dipalmitoylphosphatidylcholine (DPPC; Avanti Polar Lipids, Inc., Alabaster, AL), cholesterol (Avanti Polar lipids, Inc. or Sigma Chemical Corp., St. Louis, MO) and N-(carbamoyl-methoxypolyethylene glycol 2000)-1,2-distearoyl-sn-glycero-3-phosphoethanolamine-(DSPE-MPEG<sub>2000</sub>; Avanti Polar Lipids, Inc.) were dispensed into a 30 mL round bottom flask as follows for 150  $\mu$ mol of total lipid:

**TABLE 10**

**Lipid Components of DSPE-MPEG<sub>2000</sub> Liposomes**

Comprising ISIS 2503				
Component	Mole ratio	Mole %	mg lipid	mL stock lipid solution
DPPC	3	57	62.74	3.137
Cholesterol	2	38	22.03	1.102
DSPE-MPEG <sub>2000</sub>	0.265	5	20.75	1.037

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Chloroform was removed by evaporation using a rotary evaporator, heating at 60°C with a moderate vacuum. The lipid material dried as a thin film on the flask wall. Evaporation was continued using high vacuum for an additional 30 minutes  
5 at 60°C.

#### **B. Lipid hydration**

Phosphorothioate oligonucleotide (ISIS 2503) was dissolved in water to 100 mg/mL. The solution was made isotonic (80-310 mOsm) with the addition of a small quantity  
10 of 5M NaCl as needed. The final solution was filtered through a 0.22 µm membrane. Then, 0.5 mL of the resultant oligo solution was added to the flask containing the lipid film. The flask was rotated at 240 rpm at 60°C for 5 minutes. The lipid suspension was vortexed heavily to form large multi-  
15 lamellar liposomes.

The liposomes were frozen by immersing the flask into a dry ice/acetone bath for 5 minutes. Thawing of the liposomes was accomplished by immersing the flask into a 60°C water bath as necessary. The preceding freeze/thaw steps were  
20 repeated 5 times. The resulting liposome solution appeared "creamy".

#### **C. Particle sizing**

Large multi-lamellar liposomes were converted into near-uniform unilamellar liposomes by either (1) physical extrusion  
25 through polycarbonate membranes (Avestin, Inc., Ottawa, Ontario, Canada) of defined porosity (e.g., 100 nm) or microfluidization with a Model 110 S microfluidizer (Microfluidics International Corp., Newton, MA). Either technique produces unilamellar liposomes of approximately 90  
30 to about 110 nm in diameter.

#### **D. Liposome purification**

Nonencapsulated oligonucleotide material was separated from the liposomes by gel permeation chromatography using a Superdex-200 column (Pharmacia Biotech, Inc., Piscataway, NJ)

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equilibrated in phosphate-buffered saline, pH 7.4. Encapsulation recovery was typically 25-30% and the final ISIS 2503 concentration in the liposomes was about 7 mg/mL. The liposome fractions were pooled and filter-sterilized through  
5 a 0.2  $\mu$ m membrane (Gelman Sciences, Inc., Ann Arbor, MI). Liposomes were stored at 4°C.

**EXAMPLE 4: Evaluation of Sterically Stabilized Liposomes  
Comprising Antisense Oligonucleotides**

**A. Experimental Design and Methods**

10       **Study Design:** Thirteen rhesus monkeys (*Macaca mulatta*) (7 males and 6 females) were used. The animals were pre-pubertal to young adult (in the age range of 3-7 years), and their body weight ranged from 3-4 kg (Table 11). Each animal received a single intravenous infusion of ISIS 2503  
15 encapsulated in sterically stabilized liposomes (10 mg/kg) over approximately 30 minutes. Blood samples for pharmacokinetic analysis were collected prior to dosing and at 0, 1, 2, 6, 12, 24, 40, 60, 96, 120, 144, 168, 192, 240, 384 and 576 hours after dosing. Animals were serial-  
20 sacrificed such that 2 animals (1 male and 1 female) were euthanized at each of the following time points from the end of infusion: 24, 60, 120, 168, 384 and 576 hours. An additional male monkey (Animal ID #R4791) died of unknown causes shortly after dosing. Although samples were analyzed  
25 for this animal, the values were not included in the pharmacokinetic analysis because the animal died before the earliest study time point. As controls in some experiments, animals were treated in the same manner but with a simple saline formulation of ISIS 2503 in saline.

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TABLE 11

## Animals Assigned to Study

	Animal ID	Gender	Body (kg)	Weight	Time (hr)	Point
	R4759	M	3.2		24	
5	R3524	F	4.4		24	
	R4797	M	3.7		60	
	R2700	F	3.5		60	
	R4778	M	3.5		120	
	R4784	F	3.6		120	
10	R4758	M	3.6		168	
	R4781	F	3.6		168	
	R4796	M	3.1		384	
	R4782	F	3.5		384	
	R4764	M	4.2		576	
15	R4768	F	3.5		576	

A full necropsy was conducted on all animals. The following tissues were collected from each animal: brain, heart, pancreas, prostate, ovaries, spleen, intestine, kidney cortex, kidney medulla, liver, mesenteric and mandibular  
 20 (combined, M & M) lymph nodes, axillary and inguinal (combined, A & I) lymph nodes, lung, back skin, and hand skin. Whole blood and tissue samples were extracted and analyzed by capillary gel electrophoresis (CGE).

**Sample Extraction in Whole Blood:** Blood samples were  
 25 vortexed and an aliquot (100 µl) was measured into a 2 mL Fastprep tube (BIO101, Inc., Vista, CA) containing approximately 1/4 inch of homogenization beads. Following the addition of 390 µL PBS, 5 µL 10% NP-40, and 5 µL 100 µM T<sub>27</sub> (a 27-mer phosphorothioate oligodeoxythymidine used as the



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internal standard), the mixture was homogenized in a Savant Tissue Disrupter (BIO101, Inc., Vista, CA). The samples were then extracted with phenol-chloroform to remove proteins and lipids; oligonucleotides remained in the aqueous phase. To  
5 enhance the separation of the aqueous phase from the organic phase, an aliquot of phase lock gel (Intermountain Scientific Corp., Kaysville, UT) was added to the samples after adding phenol-chloroform. The phenol-chloroform layer was back-extracted with 500  $\mu$ L of water and the aqueous phases were  
10 pooled. The aqueous phase was then evaporated to dryness, re-suspended with 5 mL SAX loading buffer (containing 10 mM Tris-HCl, 0.5 M KCl, and 20% acetonitrile, at pH 9.0) in preparation for solid phase extraction.

**Sample Extraction in Tissue:** The method for tissue  
15 sample extraction combined the proteinase K digestion method previously used for extraction of oligonucleotides from tissues (Cossum et al., *J. Pharmacol. Exp. Therap.* 269:89, 1994) with the solid phase extraction method (Leeds et al., *Analytical Biochem.* 235:36, 1996). Monkey tissues were  
20 weighed, homogenized in a Bio Savant, and incubated for 24 hours at 37°C in a 2.0 mg/mL proteinase K solution of digestion buffer consisting of 0.5% Non-Idet P-40 (NP-40) with 20 mM Tris-HCl (pH 8.0), 20 mM EDTA, and 100 mM NaCl. An appropriate amount of  $T_2$ , ranging from 0.5 to 10  $\mu$ M, was added  
25 for quantitation by capillary electrophoresis. The aqueous layer was then extracted with phenol-chloroform, the phenol-chloroform layer was back-extracted with 500  $\mu$ L of water and the aqueous phases were pooled. The aqueous layer was extracted again with chloroform to remove the phenol. Samples  
30 were then evaporated to dryness, resuspended in 200  $\mu$ l concentrated ammonium hydroxide and incubated at 55°C for 12 to 24 hours. The samples were then re-evaporated to dryness, resuspended with 5 mL SAX loading buffer (containing 10 mM

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Tris-HCl, 0.25 M KCl, and 20% acetonitrile, at pH 9.0) in preparation for solid phase extraction.

**Solid Phase Extraction:** After phenol-chloroform extraction, both blood and tissue samples were further  
5 extracted using a J&W Scientific, Inc. (Folsom, CA) strong anion exchange (SAX) SPE column. For solid phase extraction, the column was prepared for use by wetting it with 1 ml of acetonitrile followed by 1 ml of distilled water. The column was then equilibrated with 3 ml of loading buffer prior to  
10 loading the tissue or blood extracts. After loading the extracts, the anion exchange SPE column was washed with 3 mL of the loading buffer, and the oligonucleotides were eluted with 3 mL of elution buffer (containing 10 mM Tris-HCl, 0.5 M KCl, and 1.0 M NaBr, and 30% acetonitrile, at pH 9.0). The  
15 eluted samples were diluted and were then desalted using a reversed-phase solid phase extraction column.

The reversed-phase solid phase extraction column (Isolute, from Alltech Associates, Inc., Deerfield, IL) was pre-equilibrated with 1 mL acetonitrile, 1 mL distilled water,  
20 and 3 mL eluting buffer (10 mM Tris-HCl, 0.5 M KCl, and 1.0 M NaBr, at pH 9.0). After the diluted eluate from the anion exchange column was loaded onto reverse phase SPE column, it was washed with 5 mL of distilled water, and purified oligonucleotide was then eluted using 3 mL of fresh 20%  
25 acetonitrile in distilled water. After evaporation to dryness, the samples were resuspended in 40 µl distilled water, and a 15 µl aliquot was desalted by dialysis on a Millipore VS membrane (pore size 0.025 microns, Millipore Corp., Bedford, MA) floating in a 60 × 15 mm polystyrene  
30 petrie dish (Becton Dickinson and Co., Lincoln Park, NJ) containing distilled water prior to loading into microvials for analysis by capillary electrophoresis.

**Capillary Electrophoresis:** A Beckman P/ACE Model 5010 capillary electrophoresis instrument (Beckman Instruments,

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Inc., Fullerton, CA) was used for gel-filled capillary electrophoresis analysis. Samples were electrokinetically injected using an applied voltage between 3-10 kV for a duration ranging from 3-20 seconds. Length-based separation of the oligonucleotides was achieved by using a coated-capillary (Bio-Rad Laboratory, Hercules, CA) with Beckman eCAP ssDNA 100-R Gel. Separation was optimized using a constant applied voltage of 20 kV and a temperature of 40°C. Oligonucleotide peaks were detected by UV absorbance at 260 nm. Beckman System Gold Software on the P/ACE instrument was used to determine the areas under the curve for oligonucleotide peaks in the resultant electropherograms. A peak area threshold of 0.01 area units and minimum peak width of 0.08 min were the standard integration parameters (Leeds et al., *Analytical Biochem.* 235:36, 1996).

**Quantitation:** Quantitation of intact ISIS 2503 and metabolites for whole blood samples was based on the calibration curve with  $T_{27}$  as the internal standard. The limit of quantitation for this assay has been estimated to be 0.10 µg/mL oligonucleotide in blood. In contrast, the concentrations of ISIS 2503 and metabolites in the tissue samples were calculated from the ratio of the absorbencies, based only on the starting concentration of internal standard ( $T_{27}$ ) added to the samples using the following equation:

$$C_2 = C_1 (E_1/E_2) [(A_2/T_{m2}) / (A_1/T_{m1})]$$

Where  $C_1$  = concentration of the internal standard,  $C_2$  = concentration of the analyte (ISIS 2503 or metabolites),  $E_1$  = molar extinction coefficient of the internal standard,  $E_2$  = molar extinction coefficient of the analyte,  $A_1$  = area of the internal standard peak,  $A_2$  = area of the analyte peak,  $T_{m1}$  = migration time of the internal standard peak, and  $T_{m2}$  = migration time of the analyte peak.

Calculations of extinction coefficients for ISIS 2503, metabolites, and  $T_{27}$ , are made using a program which calculates

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the sums of the extinction coefficients from the individual bases according to the base composition. For the calculation of extinction coefficients, metabolites are assumed, to be generated by loss of nucleotide from the 3'-end. The limit  
5 of quantitation for this assay has been estimated to be 0.10 µg/g oligonucleotide in tissue.

**Pharmacokinetic Analysis:** Inspection of the semi-logarithmic plots of intact ISIS 2503 (full length) blood level-versus time curves indicated that they could be  
10 described by a monoexponential equation. First order elimination was assumed. Initial estimates of parameters were obtained by linear regression of the terminal concentration time points. Nonlinear regression was accomplished using a one compartment model for each individual animal (WinNonlin  
15 1.0, Scientific Consulting, Inc., Apex, NC). A uniform weight of 1 was used for all blood-level data. Four of the animals were excluded from complete individual pharmacokinetic analysis of blood concentrations because they were sacrificed before a complete blood profile could be collected (2 at 24  
20 hours and 2 at 60 hours).

Tissue elimination was analyzed by noncompartmental methods using WinNonlin 1.0. Tissue half-lives were estimated by linear regression analysis of the log-linear terminal phase of the tissue concentration-time curve. The area under the  
25 tissue concentration-time curve ( $AUC_{0-\infty}$ ) and the area under the first moment of the concentration-time curve ( $AUMC_{0-\infty}$ ) were calculated using the linear trapezoidal rule, up to the last measured time point, plus the extrapolated area. The mean residence time (MRT) was calculated as the ratio of the  
30  $AUMC_{(0-\infty)}$  to the  $AUC_{(0-\infty)}$ .

**Statistics:** Statistical analysis for gender difference of kinetic parameters was performed by F-test (Excel 6.0, Microsoft Corp., Redmond, WA) for the analysis of variance, and t-test (Excel 6.0) for the analysis of mean at the  $p =$

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0.05 level. Descriptive statistics were used to present data summaries for pharmacokinetic parameter estimates and blood concentration data.

## B. Results

5        **Blood Pharmacokinetics and Metabolism:** Figures 6 and 7 are representative electropherograms of (a) liposomal and (b) saline formulations of ISIS 2503 in blood and kidney samples, respectively, from monkeys after i.v. infusions of 10 mg/kg of the respective formulations. The saline  
10 formulated oligonucleotide samples were taken either 1 hour after initiation of a 2-hour infusion in the case of plasma or 48 hours after the last 2-hour infusion of 14 doses administered every other day (q2d). In contrast, the liposomal oligonucleotide formulations were evaluated at 60  
15 hours after an 0.5 hour infusion. Despite the longer period during which the liposomal oligonucleotide formulations were exposed to degradative processes in the tissues, the ISIS 2503 remained in a significantly more intact state than the saline-formulated oligonucleotide (as can be seen by comparing panel  
20 (a) in Figures 6 and 7 to panel (b)).

The time course of the clearance of ISIS 2503 and oligonucleotide metabolites from blood after administration of the liposomal oligonucleotide composition is prolonged (see Tables 12 and 13 and Figure 1). Maximum blood concentration  
25 ( $C_{max}$ ) of intact ISIS 2503 was approximately 90  $\mu\text{g/mL}$  and was observed at the end of the 30-minute infusion. ISIS 2503 concentration did not decrease by 1 hour after infusion but remained at c. 90  $\mu\text{g/mL}$ . Concentrations in blood decreased slowly to approximately 10  $\mu\text{g/mL}$  at 144 hours after infusion.  
30 In these experiments, the method for quantitating ISIS 2503 concentrations in blood or tissues does not distinguish between free and liposome encapsulated oligonucleotides, and both parent compound and total oligonucleotide concentrations are presented because many of the chain-shortened metabolites

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retain physical and chemical properties similar to those of the parent compound ISIS 2503 and thus may potentially have some biological activity.

Pharmacokinetic parameter estimates for males and females were averaged since statistical analysis indicated no significant gender differences. The mean blood half-life for intact ISIS 2503 was 57.2 hours (Table 14). The concentration of ISIS 2503 in blood generally fell below the limit of detection after 168 hours. The observed concentrations in blood were less well predicted by the model after 120 hours (Figure 1). The values predicted by the model were higher than the actual values observed at the late time points suggesting that there were alterations in the kinetics after extended circulation times. This phenomenon may be a result of the loss of liposome integrity after prolonged circulation in blood. The average total body clearance and  $V_{d_{ss}}$  were  $1.53 \pm 0.28$  mL/hr/kg and  $123 \pm 28$  mL/kg, respectively. The volume of distribution was larger than the blood compartment (73.4 mL/kg) indicating some distribution into tissues, but also indicated a large portion of administered dose remained in the general circulation (Davies et al., *Pharm. Res.* 10:1093, 1993).

The metabolites of ISIS 2503 in blood co-migrated on CGE with ISIS 2503 shortened by removal of 1 or 2 bases (19-mer and 18-mer; in Tables 12, 13 and 15 these are referred to as "n-1" and "n-2," respectively). Concentrations of metabolites observed were an order of magnitude lower than that of parent drug. The chain-shortened metabolites cumulatively represented approximately 5% to 20% of the total oligonucleotides in blood. There was only a small increase in the percentage of oligonucleotide metabolites with time. This pattern of very low concentrations of metabolites observed in blood suggests that liposomal encapsulation protected the oligonucleotide from blood (and tissue)

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nucleases that might otherwise rapidly metabolize the circulating oligonucleotide, and supports the notion that there was very little leakage of ISIS 2503 from the liposome.

TABLE 12

5      Concentrations ( $\mu\text{g/mL}$ ) of ISIS 2503 and All Detected  
Metabolites in Blood After 0.5 hr Intravenous Infusion  
of 10 mg/kg ISIS 2503 Encapsulated in Sterically  
Stabilized Liposomes to Rhesus Monkeys

10	Time (hr)	No. of Animals	Mean Concentration ( $\mu\text{g/mL}$ )			Total	% Full Length
			ISIS 2503	n-1	n-2		
	0	12	89.0 <sup>a</sup> (24.7) <sup>b</sup>	4.27 (3.54)	nd	93.3 (25.4)	95.5 (3.5)
	1	12	90.4 (18.9)	3.44 (2.24)	0.28 (0.96)	94.1 (20.0)	96.1 (2.5)
	2	12	82.1 (22.0)	3.27 (2.10)	nd	85.4 (22.2)	96.1 (2.6)
	6	12	78.3 (20.4)	2.92 (1.64)	0.31 (1.06)	81.5 (20.6)	96.0 (2.6)
15	12	12	64.6 (18.7)	1.35 (1.58)	0.28 (0.96)	66.2 (19.7)	97.8 (2.9)
	24	12	63.3 (18.2)	2.08 (1.26)	nd	65.4 (18.3)	96.7 (2.2)
	40	10	50.1 (15.0)	1.45 (1.02)	0.06 (0.17)	51.9 (15.4)	96.9 (1.8)
	60	10	50.6 (9.5)	1.37 (1.02)	0.29 (0.91)	56.7 (16.6)	92.2 (14.0)
	96	8	22.6 (12.9)	2.65 (2.23)	nd	25.3 (14.6)	91.9 (7.2)
			15.7	2.61		18.3	89.2

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120	7	(10.2)	(1.91)	nd	(12.0)	(8.0)
		10.6	1.07		11.7	94.2
144	4	(6.3)	(2.14)	nd	(7.4)	(11.6)
		4.42	0.40		4.82	93.5
	4	(1.32)	(0.69)	nd	(1.67)	(11.3)
168						
		3.62	0.59		4.21	84.4
192	2		(0.83)	nd	(0.60)	(22.0)
		2.13	1.19		3.32	64.2
240	1			nd		

5 "% Full-length" = percent of total detectable oligonucleotide represented by intact ISIS 2503.

"nd" = not detected (detection level = 0.10 µg/mL).

<sup>a</sup> Mean value.

<sup>b</sup> Standard deviation.

10

TABLE 13

ISIS 2503 and Total Oligonucleotide Whole Blood  
Concentrations after 0.5 hr i.v. Infusion of 10 mg/kg ISIS 2503  
Encapsulated in Sterically Stabilized Liposomes to  
Rhesus Monkeys (Average of Duplicate Analysis)

15	Animal Gender Time <sup>a</sup> µg/mL							
	ID #	(hr)	( ISIS 2503	n-1	n-2	n-3	Total	% Full <sup>b</sup>
	R4759 M	0	115	1.06	nd	nd	116	99.1
	R4759 M	1	120	6.58	3.32	nd	130	92.4
	R4759 M	2	93.8	6.11	nd	nd	99.9	93.9
20	R4759 M	6	89.8	5.71	3.66	nd	99.2	90.6



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	R4759	M	12	78.7	5.30	3.33	nd	87.3	90.1
	R4759	M	24	58.9	4.09	nd	nd	63.0	93.5
	R3524	F	0	131	1.11	nd	nd	132	99.2
	R3524	F	1	92.2	0.33	nd	nd	92.5	99.6
5	R3524	F	2	92.9	0.58	nd	nd	93.5	99.4
	R3524	F	6	102	0.90	nd	nd	103	99.1
	R3524	F	12	98.8	0.77	nd	nd	99.6	99.2
	R3524	F	24	76.4	0.11	nd	nd	76.5	99.9
	R4797	M	0	84.5	6.33	nd	nd	90.8	93.0
10	R4797	M	1	85.1	6.42	nd	nd	91.5	93.0
	R4797	M	2	71.4	6.30	nd	nd	77.7	91.9
	R4797	M	6	66.1	3.65	nd	nd	69.8	94.8
	R4797	M	12	68.5	nd	nd	nd	68.5	100
	R4797	M	24	53.8	3.17	nd	nd	56.9	94.4
15	R4797	M	40	40.4	0.89	nd	nd	41.3	97.8
	R4797	M	60	51.4	0.49	nd	nd	96.3	53.4
	R2700	F	0	75.7	6.61	nd	nd	82.3	92.0
	R2700	F	1	82.1	6.62	nd	nd	88.7	92.5
	R2700	F	2	64.9	6.15	nd	nd	71.1	91.3
20	R2700	F	6	68.8	6.03	nd	nd	74.8	91.9
	R2700	F	12	45.1	nd	nd	nd	45.1	100

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	R2700	F	24	58.7	1.48	nd	nd	60.2	97.5
	R2700	F	40	44.4	1.34	0.55	nd	46.3	95.9
	R2700	F	60	61.3	0.72	nd	nd	62.0	98.8
	R4778	M	0	96.3	10.3	nd	nd	107	90.3
5	R4778	M	1	124	1.58	nd	nd	126	98.7
	R4778	M	2	133	2.59	nd	nd	135	98.1
	R4778	M	6	128	2.78	nd	nd	131	97.9
	R4778	M	12	94.7	2.60	nd	nd	97.3	97.3
	R4778	M	24	106	2.05	nd	nd	108	98.1
10	R4778	M	40	44.6	1.52	nd	nd	46.1	96.7
	R4778	M	60	42.3	1.03	nd	nd	43.4	97.6
	R4778	M	96	5.03	nd	nd	nd	5.03	100
	R4784	F	0	92.9	10.5	nd	nd	103	89.8
	R4784	F	1	89.6	1.94	nd	nd	91.5	97.9
15	R4784	F	2	95.0	1.96	nd	nd	96.9	98.0
	R4784	F	6	85.0	1.34	nd	nd	86.3	98.5
	R4784	F	12	45.7	nd	nd	nd	45.7	100
	R4784	F	24	70.9	2.05	nd	nd	72.9	97.2
	R4784	F	40	48.3	nd	nd	nd	91.5	97.9
20	R4784	F	60	53.4	0.97	nd	nd	54.3	98.2
	R4784	F	96	43.5	3.99	nd	nd	47.4	91.6

	R4784	F	120	23.3	3.45	nd	nd	26.8	87.1
	R4758	M	0	109	4.91	nd	nd	114	95.7
	R4758	M	1	78.1	3.08	nd	nd	81.2	96.2
	R4758	M	2	69.0	2.60	nd	nd	71.6	96.4
5	R4758	M	6	75.1	2.99	nd	nd	78.1	96.2
	R4758	M	12	62.4	0.87	nd	nd	63.3	98.6
	R4758	M	24	85.6	3.35	nd	nd	89.0	96.2
	R4758	M	40	48.1	1.22	nd	nd	49.3	97.5
	R4758	M	60	52.1	0.71	nd	nd	52.8	98.7
10	R4758	M	96	32.2	4.78	nd	nd	37.0	87.1
	R4758	M	120	18.3	2.48	nd	nd	20.8	88.1
	R4758	M	144	17.3	nd	nd	nd	17.3	100
	R4758	M	168	11.8	nd	nd	nd	11.8	100
	R4781	F	0	90.6	4.07	nd	nd	94.6	95.7
15	R4781	F	1	96.1	4.24	nd	nd	100	95.8
	R4781	F	2	105	5.26	nd	nd	110	95.2
	R4781	F	6	67.1	3.12	nd	nd	70.2	95.6
	R4781	F	12	43.9	nd	nd	nd	43.9	100
	R4781	F	24	53.4	2.66	nd	nd	56.0	95.3
20	R4781	F	40	51.8	1.57	nd	nd	53.4	97.1
	R4781	F	60	52.9	0.85	nd	nd	53.8	98.4

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	R4781	F	96	26.0	4.48	nd	nd	30.5	85.3
	R4781	F	120	15.5	4.28	nd	nd	19.7	78.3
	R4781	F	144	3.46	nd	nd	nd	3.46	100
	R4781	F	168	2.93	nd	nd	nd	2.93	100
5	R4782	M	0	30.9	0.30	nd	nd	31.2	99.1
	R4782	M	1	72.4	1.42	nd	nd	73.8	98.1
	R4782	M	2	60.4	1.16	nd	nd	61.5	98.1
	R4782	M	6	62.1	1.32	nd	nd	63.4	97.9
	R4782	M	12	58.3	1.05	nd	nd	59.4	98.2
10	R4782	M	24	51.8	0.89	nd	nd	52.7	98.3
	R4782	M	40	37.5	2.56	nd	nd	40.1	93.6
	R4782	M	60	43.8	3.84	2.89	nd	50.5	86.7
	R4782	M	96	26.8	3.50	nd	nd	30.3	88.5
	R4782	M	120	19.1	3.52	nd	nd	22.6	84.4
15	R4782	M	144	14.1	4.27	nd	nd	18.4	76.8
	R4782	M	168	5.45	nd	nd	nd	5.45	100
	R4782	M	192	4.64	nd	nd	nd	4.64	100
	R4796	F	0	76.1	2.70	nd	nd	78.8	96.6
	R4796	F	1	106	4.74	nd	nd	111	95.7
20	R4796	F	2	64.1	2.36	nd	nd	66.4	96.5
	R4796	F	6	73.4	3.23	nd	nd	76.6	95.8

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	R4796	F	12	63.0	1.40	nd	nd	64.4	97.8
	R4796	F	24	49.1	0.89	nd	nd	50.0	98.2
	R4796	F	40	43.1	2.13	nd	nd	45.2	95.3
	R4796	F	60	43.7	1.21	nd	nd	44.9	97.3
5	R4796	F	96	21.1	4.44	nd	nd	25.5	82.6
	R4796	F	120	29.3	4.58	nd	nd	33.8	86.5
	R4796	F	144	7.42	nd	nd	nd	7.42	100
	R4796	F	168	4.89	1.19	nd	nd	6.08	80.4
	R4796	F	192	2.61	1.18	nd	nd	3.79	68.9
10	R4796	F	240	2.13	1.19	nd	nd	3.32	64.2
	R4768	F	0	80.5	1.68	nd	nd	82.1	98.0
	R4768	F	1	76.4	1.35	nd	nd	77.8	98.3
	R4768	F	2	72.2	1.30	nd	nd	73.5	98.2
	R4768	F	6	69.6	1.46	nd	nd	71.0	97.9
15	R4768	F	12	69.4	1.31	nd	nd	70.7	98.1
	R4768	F	24	50.4	0.84	nd	nd	51.3	98.4
	R4768	F	40	90.6	3.25	nd	nd	93.8	96.5
	R4768	F	60	68.7	2.44	nd	nd	71.1	96.6
	R4768	F	96	5.61	nd	nd	nd	5.61	100
20	R4768	F	120	3.24	nd	nd	nd	3.24	100
	R4764	M	0	85.7	1.64	nd	nd	87.4	98.1
	R4764	M	1	61.8	2.99	nd	nd	64.8	95.4

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	R4764	M	2	63.9	2.89	nd	nd	66.8	95.7
	R4764	M	6	52.8	2.57	nd	nd	55.4	95.4
	R4764	M	12	46.7	2.88	nd	nd	49.6	94.2
	R4764	M	24	44.5	3.35	nd	nd	47.8	93.0
5	R4764	M	40	52.0	nd	nd	nd	52.0	100
	R4764	M	60	37.0	1.40	nd	nd	38.4	96.4
	R4764	M	96	20.8	nd	nd	nd	20.8	100
	R4764	M	120	1.16	nd	nd	nd	1.16	100

"nd" = not detected

10     <sup>a</sup> Time is given in hours.

<sup>b</sup> "%Full" = % full-length oligonucleotide detected.

TABLE 14

Summary of Estimated Pharmacokinetic Parameters (n=8) for  
 ISIS 2503 (10 mg/kg) Encapsulated in Sterically Stabilized  
 15 Liposomes Administered to Rhesus Monkeys by 0.5 hr Infusion

	Parameter	Mean	SD	CV % <sup>b</sup>
	AUC ( $\mu\text{g}\cdot\text{hr}/\text{mL}$ )	6760	1240	18.4
	$K_{10}-t_{1/2}$ (hr)	57.2	14.2	24.9
	C <sub>max</sub> ( $\mu\text{g}/\text{mL}$ ) <sup>a</sup>	90.4	23.0	26.9
20	Cl (mL/hr/kg)	1.52	0.28	18.3
	MRT (hr)	82.5	20.5	24.9
	V <sub>d<sub>ss</sub></sub> (mL/kg)	123	28	22.3

<sup>a</sup> Data obtained from 12 animals.

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<sup>b</sup> CV% = Coefficient of Variation = (Standard deviation / Mean) x 100.

In sum, encapsulation of phosphorothioate oligonucleotide into liposomes greatly modified oligonucleotide pharmacokinetics. ISIS 2503 in liposomes was cleared slowly from the blood compared with previous experience with unencapsulated oligonucleotide. Phosphorothioate oligonucleotide concentration following intravenous infusion of unencapsulated oligonucleotide in monkeys decreases rapidly from circulation with an average distribution half-life of 36-83 minutes (Agrawal et al., *Clinical Pharmacokinet.* 28:7, 1995). In contrast, the distribution phase half-life of ISIS 2503 in this liposome formulation was markedly longer (approximately 57 hours), and resulted in an AUC that was approximately 70-fold greater than an equivalent dose of an unencapsulated oligonucleotide.

**Tissue Distribution, Elimination Kinetics and Metabolism:**  
ISIS 2503 was distributed widely into all the tissues analyzed. The highest tissue concentrations of total oligonucleotide were measured in liver, with slightly lower concentrations detected in spleen, followed by the lymph nodes, lung, hand skin, kidney cortex and medulla, heart, back skin, pancreas, colon, and brain (Table 15, Figures 2-5). It appears that the primary organs of ISIS 2503 distribution were the organs of the reticulo-endothelial system. Largest sample to sample variability was observed in skin where, presumably, the thickness of the skin layer collected varied greatly. Tissue distribution was also greatly different for the liposome formulation compared with unencapsulated oligonucleotides studied previously (Agrawal et al., *Clinical Pharmacokinet.* 28:7, 1995; Cossum et al., *J. Pharmacol. Exp. Therap.* 267:1181, 1993), where the highest concentration of oligonucleotide is consistently observed in kidney.

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Relatively long half-lives of ISIS 2503 were observed in all tissues studied (Table 16). The mean residence time (15 days) of ISIS 2503 in the kidney cortex was the longest among all the tissues examined. This slow clearance may represent  
 5 slow metabolism in the kidney or, alternatively, the kidney may take up free oligonucleotide from the circulation as it is slowly released from liposomes, thus giving the appearance of prolonged half-life. Uptake was slow in all tissues with a time to peak concentration from 1 to 7 days. The concentration  
 10 of ISIS 2503 in brain, prostate, and ovaries was still increasing up to seven days after dosing. However, the concentration of ISIS 2503 in these tissues was below the limit of quantitation for the CGE analysis by 384 hours (the next data point after the 7-day time point).

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TABLE 15

**Average (n=2) Tissue Concentrations (µg/g) of ISIS 2503 and All Detected Metabolites After 0.5 hr Intravenous Infusion of 10 mg/kg ISIS 2503 Encapsulated in Sterically Stabilized Liposomes to Rhesus Monkeys**

20	Time (hr)	(µg/mL) 2503	n+1	n-1	n-2	n-3	n-4	n-5	n-6	Total	% Full Length
	<b>Kidney Cortex</b>										
	24	0.37	13.4	0.73	0.38	0.46	0.24	0.11	0.07	15.7	77.2
	60	0.24	11.2	0.61	0.34	0.13	0.09	0.05	0.04	12.7	88.7
25	120	0.12	4.87	0.44	0.38	0.38	0.17	0.20	0.06	6.66	72.1
	168	0.18	6.00	0.72	0.22	0.94	0.48	0.36	0.16	9.31	66.2
	384	0.13	1.97	0.36	0.43	0.52	0.39	0.22	0.10	4.35	45.3
	576	0.07	0.90	0.19	0.20	0.09	nd	nd	nd	1.45	62.1





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	60	0.04	2.80	0.07	0.03	0.03	nd	nd	nd	2.96	95.0
	120	0.03	4.63	0.16	0.06	0.06	0.02	0.01	nd	4.96	93.3
	168	0.08	11.1	0.53	0.18	0.19	0.06	0.04	0.01	12.2	93.2
	384	0.02	0.24	0.02	nd	nd	nd	nd	nd	0.26	88.7
5	576	0.01	0.09	nd	nd	nd	nd	nd	nd	0.10	89.5

**Hand Skin**

	24	0.14	13.6	0.23	0.03	0.04	0.02	0.02	0.01	14.1	96.4
	60	0.13	23.9	0.68	0.20	0.11	0.09	0.06	0.03	25.2	94.2
	120	0.17	24.6	0.92	0.22	0.29	0.08	0.04	0.03	26.4	93.3
10	168	0.32	25.7	1.02	0.27	0.39	0.17	0.21	0.07	28.3	92.2
	384	0.13	13.54	1.11	1.46	0.47	0.11	0.05	0.04	17.02	79.6
	576	0.10	1.10	0.13	0.06	0.16	0.03	nd	nd	1.56	70.5

**A & I Lymph Nodes**

	24	0.26	27.7	0.85	0.26	0.17	0.02	nd	nd	29.3	94.6
15	60	0.05	43.5	2.07	0.57	0.96	0.38	0.13	nd	47.6	91.2
	120	0.24	68.2	4.16	1.61	3.10	1.08	0.23	0.07	78.7	86.6
	168	1.03	42.4	4.05	2.03	2.99	1.30	0.41	0.36	55.4	77.0
	384	0.26	29.7	2.33	0.99	1.85	0.61	0.61	0.46	39.2	75.8
	576	0.23	14.6	0.79	0.28	0.55	0.26	0.26	0.14	18.0	81.1

20 **M & M Lymph Nodes**

	24	0.21	9.47	0.49	0.37	0.11	0.11	0.07	0.05	11.0	86.0
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	60	0.23	14.4	1.03	0.82	0.50	0.29	0.22	0.16	17.8	81.1
	120	0.22	43.8	3.18	1.38	2.19	0.65	0.31	0.18	52.0	83.9
	168	0.18	38.3	3.80	1.97	2.61	1.02	0.56	0.43	49.1	79.1
	384	0.10	15.1	1.28	0.58	1.08	0.44	0.38	0.47	21.8	69.3
5	576	0.05	8.01	0.37	0.18	0.20	0.09	0.05	0.07	9.24	88.9

**Brain**

	24	0.06	2.21	0.03	nd	nd	nd	nd	nd	2.30	96.0
	60	0.06	1.28	0.01	nd	nd	nd	nd	nd	1.35	92.5
	20	0.06	0.94	nd	nd	nd	nd	nd	nd	1.00	94.2
10	168	0.05	2.27	nd	nd	nd	nd	nd	nd	2.32	97.6

**Colon**

	24	0.05	5.44	0.13	0.05	0.01	0.01	0.01	0.01	5.71	95.2
	60	0.14	4.02	0.19	0.11	0.09	0.03	0.02	nd	4.65	86.3
	120	0.03	6.88	0.65	0.35	0.39	0.05	0.01	nd	8.35	82.3
15	168	0.08	6.48	0.47	0.55	0.06	0.05	0.04	0.01	7.75	86.8

**Heart**

	24	0.04	10.3	0.28	nd	nd	nd	nd	nd	10.6	97.2
	60	0.12	6.28	0.20	0.06	0.06	nd	nd	nd	6.72	94.2
	120	0.05	3.72	0.11	nd	nd	nd	nd	nd	3.89	95.8
20	168	0.07	2.78	0.10	0.05	nd	nd	nd	nd	2.99	91.3

**Lung**

	24	0.14	22.5	0.44	0.20	0.03	nd	nd	nd	23.3	96.6
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60	0.05	26.1	0.22	0.02	nd	nd	nd	nd	26.4	98.5
120	0.10	6.95	0.22	nd	nd	nd	nd	nd	7.28	93.8
168	0.19	4.45	0.09	0.03	0.02	0.01	nd	nd	4.79	90.5

**Pancreas**

5	24	0.11	3.63	0.14	0.20	0.35	0.36	0.72	0.60	9.41	41.2
	60	0.12	5.53	0.30	0.13	0.30	0.19	0.44	0.63	10.6	54.6
	120	0.09	2.62	0.16	0.12	0.27	0.35	0.24	0.46	4.75	53.8
	168	0.07	1.78	0.25	0.33	0.09	0.06	0.30	0.55	3.46	53.2

**Prostate**

10	24	0.11	3.02	0.01	0.03	nd	nd	nd	nd	3.13	96.5
	60	0.12	3.72	0.17	0.13	nd	nd	nd	nd	4.28	81.5
	120	0.09	2.35	0.16	0.09	0.42	0.19	0.26	0.11	3.75	64.7
	168	0.07	6.89	0.49	0.21	0.36	0.12	0.10	0.14	8.63	80.1

**Ovary**

15	24	0.09	5.18	0.20	0.16	0.13	nd	nd	nd	5.77	89.8
	60	0.52	6.93	0.29	0.17	0.11	0.06	0.01	0.03	8.15	85.1
	120	0.13	5.49	0.29	0.22	0.15	1.97	0.08	0.05	8.63	65.2
	168	0.26	6.58	0.91	0.41	0.79	0.30	0.26	0.43	10.5	62.7

nd" = not detected (detection level = 0.10 µg/mL)

TABLE 16

Estimated Tissue Pharmacokinetic Parameters for ISIS 2503 (10  
mg/kg) Encapsulated in Sterically Stabilized Liposomes  
Administered to Rhesus Monkeys by 0.5 hr Intravenous Infusion

	5	Tissue	T <sub>1/2</sub> (day)	MRT (day)	T <sub>max</sub> (day)	C <sub>max</sub> (µg/g)
		Kidney Cortex	11	15	1	23.3
		Kidney Medulla	5.6	8.2	1	22.6
		Liver	4.2	8.1	7	160
		A & I Lymph Node	NA <sup>a</sup>	18	5	97.0
10		M & M Lymph Node	7.7	13	5	57.4
		Spleen	9.7	14	5	107
		Back Skin	3.1	7.0	7	16.5
		Hand Skin	4.2	10	2	43.8
		Lung	2.0	3.4	2	32.3
15		Heart	3.0	5.7	1	12.7
		Pancreas	3.2	6.4	2	9.43
		Brain	NA	NA	1	2.41
		Colon	NA	NA	7 <sup>b</sup>	8.24
		Ovary	NA	NA	2	6.92
20		Prostate	NA	NA	7 <sup>b</sup>	6.89

<sup>a</sup> "NA" = not available

<sup>b</sup> Concentration was still increasing at the last analyzed time point.

The appearance of metabolites was low even 576 hours after  
25 infusion (Table 15). Very low relative percentage of

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metabolites were observed for all the organs (~10-20%) except for the liver, the kidney cortex, and the pancreas (~30-60%). Higher concentration of oligonucleotide metabolites was observed as early as 24 hours after infusion in the pancreas.

5 Although not wishing to be bound by any particular theory, this phenomenon could be related to the activity of lipases in this organ allowing more ISIS 2503 to escape from liposomes and be metabolized (McNeely et al., "Pancreas Function" In: *Clinical Chemistry: Theory, Analysis, and Correlation*, Kaplan and Pesce,

10 eds., The C.V. Mosby Company, St. Louis, pp. 390-397, 1989). At later time points ( $\geq 120$  hr), increasing concentrations of chain-shortened oligonucleotide metabolites were seen in liver and kidney. Kidney and/or liver may also play a role in the degradation of liposomes but, alternatively, may be primary

15 sites of free oligonucleotide and metabolite distribution.

In addition to chain-shortened metabolites, there were also UV absorbing peaks that migrated more slowly than parent oligonucleotide. Slower migrating oligonucleotide peaks have been identified for other phosphorothioate oligonucleotides in

20 tissue. Slower migration suggests that the mass to charge ratio was increased either from the addition of a substituent or loss of charge. These metabolites are thought to represent intact drugs plus an additional substituent possibly an additional nucleotide or two (Griffey et al., *J. Mass. Spec.*

25 32:305, 1997). Thus, while not wishing to be bound by any particular theory, it is possible that the slower migrating peak observed in these studies is such a lengthened metabolite, and this peak is thus referred to as "n+1" in Table 15.

**Toxicokinetic Summary and Conclusions:** In this

30 investigation, it has been demonstrated that ISIS 2503 in a sterically stabilized liposome formulation has a markedly prolonged circulation time. Maximum concentration ( $C_{max}$ ) in blood is achieved at the end of infusion and it is

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approximately 90 µg/mL. Pharmacokinetic modeling of ISIS 2503 indicates a slow distribution process with a half-life of approximately 57 hours. The half-life of ISIS 2503 in this formulation is significantly greater than that observed for unencapsulated oligonucleotides suggesting that ISIS 2503 in liposomes is slowly distributed to tissues and protected from metabolism in blood. Unencapsulated oligonucleotide is cleared from plasma by a combination of metabolism and tissue distribution. Unencapsulated oligonucleotide has been reported to have half-lives ranging from 36-83 minutes. With this formulation there appears to be little metabolism, and clearance from blood is slow with a half-life of 57 hours. Clearly this formulation has altered the kinetics of circulating oligonucleotide. While not wishing to be bound by any particular theory, because tissue distribution is the primary route for both liposomal oligonucleotide and unencapsulated oligonucleotide clearance from circulation, slower kinetics of liposome uptake seen in tissue may explain the prolonged circulation of oligonucleotide in this study.

Liposomal ISIS 2503 is widely distributed into all tissues tested, in descending maximum concentration ( $C_{max}$ ) order, liver> spleen> lymph nodes> hand skin> lung> kidney> back skin> heart> pancreas> colon> ovary> prostate> brain. Intact ISIS 2503 is the predominant oligonucleotide species measured indicating a slow metabolism in tissues and supporting the concept that liposomes remain intact in tissues. The apparent increase in metabolites observed in kidney, liver, and pancreas could be explained by digestion of the liposomes in these tissues, or preferential uptake of metabolites from circulation by these tissues. The high oligonucleotide concentrations in liver and spleen suggest that liposome formulations are primarily removed from blood by the reticulo-endothelial system. The persistence and abundance of intact ISIS 2503 in tissues is best explained

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by the protection from nucleases afforded by liposomal encapsulation.

**EXAMPLE 5: Evaluation of the Antitumor Activity of Sterically Stabilized Liposomes Comprising Antisense Oligonucleotides**

One advantage of some sterically stabilized liposomes is their ability to deliver conventional chemotherapeutic agents to tissues, particularly tumors, other than those of the reticuloendothelial system (RES) (Gabizon et al., *Proc. Natl. Acad. Sci. U.S.A.* 85:6949, 1988; Papahadjopoulos et al., *Proc. Natl. Acad. Sci. U.S.A.* 88:11460, 1991). In disease states where leaky vasculature is characteristic (e.g., inflammation, tumors), prolonging the circulation time via the liposomal oligonucleotide formulations of the invention may allow for more effective delivery of oligonucleotide as well as providing for a less frequent oligonucleotide dosing interval. In order to test the efficacy of liposomal oligonucleotide formulations against tumors, a human-mouse xenograft model was used.

**A. Experimental Design and Methods**

**Liposomes:** Sterically stabilized liposomes comprising DSPE-MPEG<sub>2000</sub> in the lipid phase and ISIS 2503 in the aqueous phase were prepared as in Example 3. ISIS 2503 loaded sterically stabilized liposomes comprising the monosialoganglioside G<sub>M1</sub> instead of DSPE-MPEG<sub>2000</sub> were prepared in like fashion, except that monosialoganglioside G<sub>M1</sub> (Sigma Chemical Co., St. Louis, MO) was substituted for DSPE-MPEG<sub>2000</sub> at the same final molar concentration. In some experiments, ISIS 13177 was used as a control. This phosphorothioate oligodeoxynucleotide has the nucleotide sequence 5'-TCAGTAATAGCCCCACATGG (SEQ ID NO: 26). In other experiments, ISIS 2105 was used as a control. This oligonucleotide has the nucleotide sequence 5'-TTGCTTCCATCTTCCTCGTC (SEQ ID NO: 27), which is targeted to the E2 gene of papillomavirus HPV-11.



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Saline formulations of ISIS 2503 were also included as controls in the experiments.

**Xenografts:** Xenografts of human tumor cell lines into BALB/c nude mice were performed essentially as described by Dean et al. (*Cancer Res.* 56:3499, 1996). Cell lines NCI-H69 and MIA PaCa-2 are available from the American Type Culture Collection (A.T.C.C., Rockville, MD) as accession numbers ATCC HTB-119 and ATCC CRL-1420, respectively.

**Dosing and Analysis:** Formulations were administered intraperitoneally (i.p.) or intravenously (i.v.) at the indicated frequencies including every other day (q2d) and every third day (q3d). Tumor volume was measured at the indicated times by measuring perpendicular diameters and calculated as described Dean et al., *Cancer Res.* 56:3499, 1996). For distribution studies, mice were given two doses of 10 mg/kg of formulation and then sacrificed after 24 hours. Tumor tissue was removed and analyzed by capillary electrophoresis for the presence of various oligonucleotide species as described in Example 4.

## 20 B. Results

**Distribution:** Sterically stabilized liposomes comprising either DSPE-MPEG<sub>2000</sub> (PEG) or monosialoganglioside G<sub>M1</sub> (GM1) resulted in enhanced delivery of ISIS 2503 to H69 and Mia PaCa tumor cells (Tables 17 and 18, respectively). The enhanced delivery was observed both in terms of increased concentration and total amount of oligonucleotide delivered to tumor tissue, and as a percentage of the total dose of oligonucleotide administered to each animal. Of particular significance is the fact that significant improvements in the percentage of intact oligonucleotide delivered to the tumor tissue increased from less than about 4% (saline formulation) to about 11% (liposomes with G<sub>M1</sub>) to over 15% (liposomes with PEG) (Table 18).

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TABLE 17

**Distribution of ISIS 2503 in Tumors in Mice  
with H69 Xenografts 24 Hours After Two Doses of 10 mg/kg**

5	Formulation	n <sup>a</sup>	Conc. (ug/g)		Amount (ug)		% of Dose	
			Avg. <sup>b</sup>	SD <sup>c</sup>	Avg.	SD	Avg.	SD
	Saline	3	3.51	0.47	1.35	0.40	0.29	0.11
	Liposomes-PEG	2	17.82	7.37	4.82	3.61	1.11	0.91
	Liposomes-GM1	2	10.01	3.24	5.40	3.85	3.08	2.08
	<sup>a</sup> "n" = number of animals.							
10	<sup>b</sup> "Avg." = average (mean).							
	<sup>c</sup> "SD" = standard deviation.							

TABLE 18

**Distribution of ISIS 2503 in Tumors in Mice with MIA PaCa  
Xenografts 24 Hours After Two Doses of 10 mg/kg**

		Conc. (µg/g)		Amount (µg)		% of Dose		
Formulation	n	Avg.	SD	Avg.	SD	Avg.	SD	
<b>A. Total concentration of ISIS 2503 &amp; metabolites</b>								
20	Saline	3	3.82	1.96	3.74	2.32	0.09	0.06
	Liposomes-PEG	3	16.21	4.98	15.27	10.93	0.36	0.25
	Liposomes-GM1	3	15.80	8.99	10.69	2.96	0.26	0.06
<b>B. Concentration of full-length ISIS 2503</b>								
25	Saline	3	0.87	0.12	0.09	0.06	3.74	3.74
	Liposomes-PEG	3	9.95	2.75	0.36	0.25	15.27	15.27
	Liposomes-GM1	3	8.56	5.18	0.26	0.06	10.69	10.69

**Antitumor Activity:** The liposomal oligonucleotide formulations of the invention were evaluated for their ability to control the growth of human tumor cells transplanted into BALB/c nude mice. One such experiment, in which liposomes comprising ISIS 2105 were used as a control formulation, is shown in Table 19.

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TABLE 19

**Antitumor Activity of Liposomal Formulations  
of ISIS 2503 Against MIA PaCa Xenografts**

				Tumor Size (mm <sup>3</sup> )		
5	Formulation:	Day	n	Mean	SD	Std. Error
<u>Saline/no oligonucleotide</u>						
		10	8	0.115	0.036	0.013
		14	8	0.321	0.119	0.042
		21	8	0.964	0.417	0.148
10		30	8	1.544	0.708	0.250
<u>PEG-Liposome/ISIS 2503 (1 mg/kg)</u>						
		10	7	0.116	0.033	0.012
		14	7	0.216	0.101	0.038
		21	7	0.700	0.335	0.127
15		30	7	1.480	0.851	0.322
<u>PEG-Liposome/ISIS 2503 (5 mg/kg)</u>						
		10	4	0.090	0.008	0.004
		14	4	0.208	0.036	0.018
		21	4	0.550	0.153	0.077
20		30	4	0.998	0.345	0.173
<u>PEG-Liposome/ISIS 2503 (25 mg/kg)</u>						
		10	6	0.102	0.047	0.019
		14	6	0.142	0.084	0.034
		21	6	0.283	0.172	0.070
25		30	6	0.603	0.331	0.135
<u>PEG-Liposome/ISIS 2105 (25 mg/kg)</u>						
		10	5	0.120	0.038	0.017
		14	5	0.294	0.180	0.081
		21	5	0.996	0.735	0.329
30		30	5	1.508	0.981	0.439

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TABLE 20

Antitumor Activity of Liposomal Formulations  
of ISIS 2503 Against NCI-H69 Xenografts

				Tumor Size (mm <sup>3</sup> )		
5	Formulation:	Day	n	Mean	SD	Std. Error
<u>Saline<sup>a</sup>/no oligonucleotide</u>						
		21	7	0.150	0.061	0.023
		28	7	0.513	0.493	0.186
		35	7	0.749	0.392	0.148
10		42	7	2.106	2.277	0.861
<u>Saline<sup>a</sup>/ISIS 2503 (25 mg/kg)</u>						
		21	7	0.163	0.056	0.021
		28	7	0.334	0.205	0.077
		35	7	0.766	0.545	0.206
15		42	7	1.021	0.751	0.284
<u>PEG-Liposome<sup>b</sup>/ISIS 2503 (25 mg/kg)</u>						
		21	6	0.150	0.068	0.028
		28	6	0.222	0.121	0.049
		35	6	0.417	0.251	0.102
20		42	6	0.753	0.551	0.225
<u>PEG-Liposome<sup>b</sup>/ISIS 13177 (25 mg/kg)</u>						
		21	7	0.163	0.043	0.016
		28	7	0.460	0.233	0.088
		35	7	0.956	0.410	0.155
25		42	7	1.636	1.037	0.392

<sup>a</sup>Saline formulations given qd.<sup>b</sup>liposomal formulations given q3d.

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In the experiment described in Table 19, sterically stabilized liposomes comprising DPSE-MPEG<sub>2000</sub> and ISIS 2503 were given in doses of 1, 5 and 25 mg/kg. Controls included a saline solution (0.9% NaCl) and sterically stabilized liposomes comprising ISIS 2105. Dosing was i.v. q3d. As can be seen in Table 19, treatment with sterically stabilized liposomes comprising ISIS 2503 resulted in a dose-dependent reduction in the rate of tumor growth. At day 21, tumor sizes averaged 0.964 and 0.996 mm<sup>3</sup> for the animals treated with, respectively, saline and liposomal ISIS 2105. In contrast, animals treated with liposomal ISIS 2503 at 1, 5 and 25 mg/kg had tumors averaging 0.700, 0.550 and 0.283 mm<sup>3</sup>, respectively.

A similar experiment (Table 20) shows that the liposomal oligonucleotide formulation is also effective against NCI-H69-derived xenografts. In this experiment, animals treated with 25 mg/kg of ISIS 2503 given as part of a liposomal formulation had tumors averaging 0.417 mm<sup>3</sup> in size on day 35, as compared to 0.749 and 0.766 mm<sup>3</sup> for saline alone and saline formulated oligonucleotide, respectively. Treatment with a liposomal formulation comprising a control oligonucleotide (ISIS 13177) at 25 mg/kg resulted in tumors averaging 0.956 mm<sup>3</sup> on day 35.

The above results demonstrate that sterically stabilized liposomal oligonucleotide formulations have several advantages over traditional formulations. First, the liposomal formulations of the invention result in improved pharmacodynamic properties (e.g., prolonged clearance time from the blood, enhanced biostability in blood and kidney samples, etc.) that result in greater circulating concentrations and stability of full-length oligonucleotides. Second, the liposomal formulations of the invention result in enhanced delivery, relative to traditional saline formulations, of the oligonucleotides encompassed thereby to tumor tissues. Third, due at least in part to the above features, liposomal oligonucleotide formulations can achieve higher concentrations

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and greater specific effects attributable to antisense oligonucleotides using a less frequent dosing regime than seen with traditional formulations (e.g., as seen from the data in Table 20, 25 mg/kg of ISIS 2503 given every third day in a liposomal formulation was more effective than the same dose of ISIS 2503 given daily in saline). Taken together, these properties are expected to result in an efficacious method for treating an animal, including a human, suffering from a hyperproliferative disease or disorder such as cancer.

10 **EXAMPLE 6:                    Pharmacokinetics        of        ISIS        2503**  
**oligonucleotide encapsulated in stealth**  
**liposomes administered rhesus monkeys**

Liposomal ISIS 2503 was formulated as 7 mg/mL oligonucleotide with a nominal lipid concentration of 15 100 mg/mL. ISIS 2503 was encapsulated using the thin-film hydration method. The lipids used were HSPC (hydrogenated soy phosphatidylcholine):mPeg-DSPE (N-(carbamoyl-methoxypolyethylene glycol 2000)-1,2-distearoyl-sn-glycero-3-phosphoethanol-amine):cholesterol at a molar ratio of 56.4:5.3: 20 38.3. ISIS 2503 was dissolved in TE/NaCl buffer pH 7.4 at 10 mg oligo/mL buffer. Particle size of 100-110 nm was achieved by extrusion through polycarbonate membranes. Sephadex G200-120 resin with TE/NaCl buffer as eluent was used to remove the unencapsulated oligo. 25-30% encapsulation was achieved, and 25 oligo concentration in the liposomes was determined by a solvent extraction method using phenol:chloroform:isoamyl alcohol (25:24:1, V/V/V).

Twelve rhesus monkeys (*Macaca mulatta*) (6 males and 6 females) were used in the liposome formulation group. These 30 animals were pre-pubertal to young adult (in the age range of 3-7 years), and their body weight ranged from 3-4 kg. Each animal received a single intravenous infusion of ISIS 2503 encapsulated in liposomes (10 mg/kg) over approximately 30

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minutes. Blood samples for pharmacokinetic analysis were collected prior to dosing and at 0, 1, 2, 6, 12, 24, 40, 60, 96, 120, 144, 168, 192, 240, 384 and 576 hours after dosing. Two animals (1 male and 1 female) were serially euthanized at  
5 each of the following time points from the end of infusion: 24, 60, 120, 168, 384 and 576 hours. A full necropsy was conducted on all animals. The following tissues were collected from each animal: brain, heart, pancreas, prostate, ovaries, spleen, intestine, kidney cortex, kidney medulla, liver, mesenteric and  
10 mandibular (combined, M & M) lymph nodes, axillary and inguinal (combined, A & I) lymph nodes, lung, back skin, and hand skin. Both whole blood and tissue samples were extracted and analyzed by capillary gel electrophoresis.

In a separate study, six rhesus monkeys (3 males and 3  
15 females) received a 2-hour i.v. infusion of 10 mg/kg ISIS 2503 formulated in saline solution every-other-day for 28 days. Blood samples were collected in EDTA tubes pre-dose, at 1 and 2 hrs during infusion, and at 1, 2 and approximately 24 hrs after the end of the infusion of the first dose. Monkeys were  
20 sacrificed 48 hours after the last dose. The following tissues were collected from each animal: heart, pancreas, spleen, kidney cortex, kidney medulla, liver, axillary and inguinal lymph nodes, lung, colon, bone marrow, prostate, ovaries, and uterus. Both plasma and tissue samples were extracted and  
25 analyzed by capillary gel electrophoresis.

Prior to sample extraction, an appropriate amount of the internal standard ( $T_{27}$ , a 27-mer phosphorothioate oligodeoxythymidine) was added to every sample. Blood samples were extracted first with phenol-chloroform. The  
30 oligonucleotides were extracted in the aqueous phase. The aqueous phase was then evaporated to dryness, re-suspended with 5 mL strong anion exchange (SAX) loading buffer (containing 10 mM Tris-HCl, 0.5 M KCl, and 20% acetonitrile, at pH 9.0) in preparation for solid phase extraction. Samples for solid

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phase extraction were processed identically as described by Leeds et al (*Anal. Biochem.*, 235:36, 1996). Plasma samples (100  $\mu$ L) were diluted with 5 mL SAX loading buffer followed by solid phase extraction as described with the following exceptions: the KCl concentration was decreased from 0.5 M to 0.25 M in the strong anion exchange running buffer, the reverse-phase solid phase extraction column used was the Glen Research Poly-Pack™ column (Glen Research, Sterling, VA) in place of the Isolute reverse-phase column, and purified oligonucleotide was eluted with 3 mL of freshly prepared 50% acetonitrile rather than 20% acetonitrile.

The method for extracting oligonucleotide from the tissues of monkeys treated with liposome formulated ISIS 2503 combined the proteinase K digestion method previously used for extraction of oligonucleotides from tissues with solid phase extraction method (Leeds et al, supra). Monkey tissues were weighed, homogenized in a Bio Savant (Bio 101, Inc., Vista, CA), and incubated for 24 hours at 37°C in a 2.0 mg/mL proteinase K (Activity: 30 units/mg, Boehringer Mannheim, Germany) solution of digestion buffer consisting of 0.5% Nonidet P-40 (NP-40) with 20 mM Tris-HCl (pH 8.0), 20 mM EDTA, and 100 mM NaCl. An appropriate amount of  $T_{25}$  was added for quantitation by capillary electrophoresis. The aqueous layer was then extracted with phenol-chloroform, the phenol-chloroform layer was back-extracted with 500  $\mu$ L of water and the aqueous phases were pooled. The aqueous layer was extracted again with chloroform to remove the phenol. Samples were then evaporated to dryness, resuspended in 200  $\mu$ L concentrated ammonium hydroxide and incubated at 55°C for 12 to 24 hours. The samples were then re-evaporated to dryness, re-suspended with 5 mL SAX loading buffer (containing 10 mM Tris-HCl, 0.25 M KCl, and 20% acetonitrile, at pH 9.0) in preparation for solid phase extraction. Samples for solid



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phase extraction were processed identically as previously described.

The method used for the extraction and analysis of the tissue samples from tissues of monkeys treated with the saline formulation was a modified procedure from that previously described, wherein proteinase K digestion and ammonium hydroxide incubation steps were omitted, and phenyl columns were used for solid phase extraction.

A Beckman P/ACE Model 5010 capillary electrophoresis instrument (Beckman Instruments, Irvine, CA) was used for gel-filled capillary electrophoresis analysis. Samples were electrokinetically injected using an applied voltage between 3-10 kV for a duration ranging from 3-20 seconds. Length-based separation of the oligonucleotides was achieved by using a coated-capillary (Bio-Rad Laboratory, Hercules, CA) with Beckman eCAP ssDNA 100-R Gel (Beckman Instruments, Fullerton, CA). Separation was optimized using a constant applied voltage of 20 kV and a 40°C temperature. Oligonucleotide peaks were detected by UV absorbance at 260 nm. Beckman System Gold Software on the P/ACE instrument was used to determine the areas under the curve for oligonucleotide peaks. A peak area threshold of 0.01 area units and minimum peak width of 0.08 min were the standard integration parameters.

Quantitation of intact ISIS 2503 and metabolites for whole blood samples was based on the calibration curve with  $T_{27}$  as the internal standard. The limit of quantitation for this assay has been estimated to be 0.10 µg/mL ISIS 2503 in blood.

In contrast, the concentrations of ISIS 2503 and metabolites in the plasma and tissue samples were calculated from the ratio of the absorbances, based on the starting concentration of internal standard ( $T_{27}$ ) added to the samples using the following equation:

$$C_2 = C_1 (E_1/E_2) [(A_2/T_{m2}) / (A_1/T_{m1})]$$

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wherein  $C_1$ =concentration of the internal standard,  
 $C_2$ =concentration of the analyte (ISIS 2503 or metabolites),  
 $E_1$ =molar extinction coefficient of the internal standard,  
 $E_2$ =molar extinction coefficient of the analyte,  $A_1$ =area of the  
5 internal standard peak,  $A_2$ =area of the analyte peak,  
 $T_{m1}$ =migration time of the internal standard peak, and  
 $T_{m2}$ =migration time of the analyte peak. Calculations of  
extinction coefficients for ISIS 2503, metabolites, and  $T_{27}$   
were made using a program that calculates the sums of the  
10 extinction coefficients from the individual bases according to  
the base composition. For the calculation of extinction  
coefficients, metabolites are assumed to be generated by loss  
of nucleotide from the 3'-end. The limit of quantitation for  
this assay has been estimated to be 0.07  $\mu\text{g/mL}$  in plasma and  
15 0.10  $\mu\text{g/g}$  in tissue.

In triplicate, encapsulated and unencapsulated ISIS 2503  
(1  $\mu\text{M}$ ) were added to 1 mL of monkey blood, respectively. After  
being centrifuged at 1500 rpm (2000 g) for 10 minutes, the  
upper plasma layer was separated from the red blood cell (RBC)  
20 layer. An aliquot of 100  $\mu\text{L}$  from each layer was extracted and  
analyzed using the method described above.  $T_{27}$  (5  $\mu\text{M}$ ) was  
added to each sample prior to sample extraction as internal  
standard.

The study of ISIS 2503 (both encapsulated and  
25 unencapsulated) distribution in blood indicated no binding or  
distribution of ISIS 2503 (both encapsulated and  
unencapsulated) on or in the red blood cells. Therefore,  
concentrations of ISIS 2503 in plasma were calculated from  
concentrations in blood by correcting for hematocrit values  
30 which for the purposes of this calculation were assumed to be  
0.41. The following equation was used:

$$[\text{ISIS 2503 Conc. in plasma}] = [\text{ISIS 2503 Conc. in blood}] / (1 - \text{hematocrit})$$

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Inspection of the semi-logarithmic plots of intact ISIS 2503 (full length) plasma concentration-versus time curves indicated that they could be described by a monoexponential equation. First order elimination was assumed. Initial estimates of  
5 parameters were obtained by linear regression of the terminal concentration time points. Nonlinear regression was accomplished using a one compartment model for each individual animal (WinNonlin 1.0). A uniform weight of 1 was used for all plasma concentration data. Four of the animals were excluded  
10 from complete individual pharmacokinetic analysis of plasma concentrations because they were sacrificed before a complete plasma profile could be collected (2 at 24 hr and 2 at 60 hr).

Tissue elimination was analyzed by noncompartmental methods using WinNonlin 1.0. Tissue half-lives were estimated  
15 by linear regression analysis of the log-linear terminal phase of the tissue concentration-time curve. The area under the tissue concentration-time curve ( $AUC_{0-\infty}$ ) and the area under the first moment of the concentration-time curve ( $AUMC_{0-\infty}$ ) were calculated using the linear trapezoidal rule, up to the last  
20 measured time point, plus the extrapolated area. The mean residence time (MRT) was calculated as the ratio of the  $AUMC_{(0-\infty)}$  to the  $AUC_{(0-\infty)}$ .

The plasma pharmacokinetics of ISIS 2503 formulated in the saline solution after single dose was analyzed by  
25 noncompartmental methods using WinNonlin 1.0. The area under the plasma concentration-time curve ( $AUC_{0-\infty}$ ) was calculated using the linear trapezoidal rule, up to the last time point, plus the extrapolated area.

Statistical analysis for gender difference of kinetic  
30 parameters was performed by F-test (Excel 6.0) for the analysis of variance, and t-test (Excel 6.0) for the analysis of mean at the  $p = 0.05$  level. Descriptive statistics were used to present data summaries for pharmacokinetic parameter estimates.

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The time course of the clearance of ISIS 2503 and oligonucleotide metabolites from plasma is greatly prolonged for the liposome formulation compared with the saline formulation (Table 21). Mean maximum plasma concentration ( $C_{max}$ ) of intact ISIS 2503 in the liposome formulation group was approximately 172  $\mu\text{g/mL}$  and was reached at the end of the 30-minute infusion. Concentrations of ISIS 2503 in plasma decreased slowly to approximately 18  $\mu\text{g/mL}$  at 144 hours after infusion. In contrast, after administration of the saline formulation, mean maximum plasma concentration ( $C_{max}$ ) of intact ISIS 2503 was approximately 65  $\mu\text{g/mL}$  and was reached at the end of the 2-hr infusion. ISIS 2503 concentration in plasma declined four-fold 2 hrs after infusion, and was below the limit of detection at 24 hrs after infusion.

Estimates of the pharmacokinetic parameters for males and females were averaged and combined since statistical analysis indicated no significant gender differences. The mean half-life in plasma for the liposome formulated ISIS 2503 was 57.8 hours, whereas the mean half-life in plasma for saline formulated ISIS 2503 was only 1.07 hours (Table 22). The average total body clearance and  $V_{d_{ss}}$  for the liposome formulation were  $0.892 \pm 0.161 \text{ mL/hr/kg}$  and  $73.0 \pm 15.3 \text{ mL/kg}$ , respectively. The average total body clearance and  $V_{d_{ss}}$  for the saline formulated ISIS 2503 were  $54.0 \pm 12.0 \text{ mL/hr/kg}$  and  $85 \pm 17 \text{ mL/kg}$ , respectively. Therefore, the total body clearance after administration of the saline formulated ISIS 2503 was over 50 times faster than after administration of the liposome formulation, indicating that ISIS 2503 in the liposome formulation was distributed and metabolized at a much slower rate compared with the saline formulation.

For the liposome formulation group, there were very small amounts of oligonucleotide metabolites observed in plasma (Table 21). A few species of chain-shortened metabolites cumulatively represented approximately less than 10% of the

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total oligonucleotides at all time points in plasma. A low level of metabolites could be observed 60 hrs after infusion of liposomal ISIS 2503. In contrast, metabolism was much more active in the saline formulation group, where the chain-  
5 shortened metabolites represented 35 to 45% of total oligonucleotides in plasma (Table 21). Moreover, more species of metabolites were also formed. A series of metabolites resulted from progressive shortening of ISIS 2503 by nucleases were observed 1 hr after the beginning of infusion.

10 After administration of ISIS 2503 in the liposome formulation, ISIS 2503 was distributed widely into all the tissues analyzed. The highest tissue concentrations of total oligonucleotide were measured in liver, with slightly lower concentrations detected in spleen, followed by the lymph nodes,  
15 lung, hand skin, kidney cortex and medulla, heart, back skin, pancreas, colon and brain. It appears that the primary organs of encapsulated ISIS 2503 distribution were the organs of the reticulo-endothelial system. Tissue distribution of liposomal ISIS 2503 differed from ISIS 2503 and other oligonucleotides  
20 in the saline formulations and other unencapsulated oligonucleotides studied previously, where the highest concentration of oligonucleotide was consistently observed in kidney cortex. Moreover, oligonucleotide concentration in kidney cortex was significantly higher than the concentration  
25 in kidney medulla for unencapsulated ISIS 2503, and this concentration difference was not observed in liposome formulated ISIS 2503. Relatively long half-lives of ISIS 2503 were observed in all tissues studied (Table 23) following administration of ISIS 2503 in liposomes. The mean residence  
30 time (15 days) of ISIS 2503 in the kidney cortex was the longest among all the tissues examined. Uptake was slow in all tissues with time to peak concentration from 1 - 7 days. The concentration of ISIS 2503 in brain, prostate, and ovaries was still increasing up to seven days post-dosing.

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For the liposome formulation group, the appearance of metabolites was low even 576 hours after infusion. Only 10 to 20% of the total oligonucleotide was in the form of metabolites in most organs. In liver, kidney cortex, and pancreas, 30 to 60% of the oligonucleotide were metabolites. Significant amounts of oligonucleotide metabolites were observed as early as 24 hours after infusion in the pancreas. At later time points ( $\geq 120$  hr), increasing concentrations of chain-shortened oligonucleotide metabolites were seen in liver and kidney. In comparison, a much higher percentage of metabolites (~62-84%) were observed in the tissues of the saline formulation group at 48 hrs from the last dose.

**TABLE 21**  
Concentrations ( $\mu\text{g/mL}$ ) of ISIS 2503 in plasma after the end of 0.5 hr (for the liposome formulation) and 2 hr (for the saline formulation) Intravenous infusion of 10 mg/kg ISIS 2503 to Rhesus Monkeys

	Time	<u>Liposome Formulation</u>		<u>Saline Formulation</u>	
		ISIS 2503 ( $\mu\text{g/mL}$ )	%Intact	ISIS 2503 ( $\mu\text{g/mL}$ )	%Intact
	0	151 $\pm$ 41.8	95.6	65.0 $\pm$ 12.5	61.0
20	1	153 $\pm$ 32.0	96.1	30.2 $\pm$ 5.7	55.3
	2	139 $\pm$ 37.3	96.1	17.4 $\pm$ 4.3	55.0
	6	133 $\pm$ 34.6	96.0	NA	NA
	12	109 $\pm$ 31.5	97.8	NA	NA
	24	107 $\pm$ 30.8	96.7	nd	nd
25	40	84.8 $\pm$ 25.2	96.8	NA	NA
	60	85.8 $\pm$ 15.9	92.2	NA	NA
	96	38.3 $\pm$ 21.8	91.9	NA	NA
	120	26.6 $\pm$ 17.3	89.2	NA	NA

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	144	17.9 ± 10.7	94.2	NA	NA
	168	10.5 ± 6.55	95.1	NA	NA
	192	6.14	84.5	NA	NA
	240	3.61	64.2	NA	NA
5	384	nd	nd	NA	NA
	576	nd	nd	NA	NA

Full length % = percent of total detectable oligonucleotide represented by intact ISIS 2503.

nd = not detected, detection level = 0.07 µg/mL.

10 NA = not applicable (samples were not collected).

TABLE 22

Summary of estimated pharmacokinetic parameters in plasma for ISIS 2503 (10 mg/kg) encapsulated in liposomes administered to Rhesus Monkeys by 0.5 hr infusion (n=8), and  
 15 in saline formulation administered to Rhesus Monkeys by 2 hr infusion (n=6)

	Parameter	<u>Liposome</u>	<u>Saline Formulation</u>
		(Mean ± SD)	(Mean ± SD)
	AUC (µg*hr/mL)	1.15×10 <sup>4</sup> ± 2.07×10 <sup>3</sup>	156 ± 30
20	t <sub>1/2</sub> (hr)	57.8 ± 13.1	1.07 ± 0.19
	C <sub>max</sub> (µg/mL)	172 <sup>a</sup> ± 33	65.0 ± 12.5
	Cl (mL/hr/kg)	0.892 ± 0.161	54.0 ± 12.0
	Vd <sub>ss</sub> (mL/kg)	73.0 ± 15.3	85.0 ± 17.0

<sup>a</sup> Data obtained from 12 animals

**TABLE 23**  
**Estimated tissue pharmacokinetic parameters for ISIS 2503**  
**(10 mg/kg) encapsulated in liposomes administered to Rhesus**  
**Monkeys by 0.5 hr intravenous infusion**

5

	Tissue	T <sub>1/2</sub> (day)	MRT (day)	T <sub>max</sub> (day)	C <sub>max</sub> (µg/g)
	Kidney Cortex	11	15	1	13.4
	Kidney Medulla	5.6	8.2	1	14.3
	Liver	4.2	8.1	7	106
10	A & I Lymph Node <sup>b</sup>	NA	18	5	68.2
	M & M Lymph Node <sup>c</sup>	7.7	13	5	43.8
	Spleen	9.7	14	5	94.0
15	Back Skin	3.1	7.0	7	11.1
	Hand Skin	4.2	10	2	25.7
	Lung	2.0	3.4	2	26.1
	Heart	3.0	5.7	1	10.3
	Pancreas	3.2	6.4	2	5.53
20	Brain	NA	NA	1	2.27
	Colon	NA	NA	7 <sup>a</sup>	6.88
	Ovary	NA	NA	2	6.93
	Prostate	NA	NA	7 <sup>a</sup>	6.89

NA = not available

25 <sup>a</sup> Concentration was still increasing at the last analyzed time point.

<sup>b</sup> Axillary and inguinal lymph node.

<sup>c</sup> Mesenteric and mandibular lymph node.



**What is claimed is:**

1. A liposomal oligonucleotide composition comprising one or more oligonucleotides, wherein each of said one or more  
5 oligonucleotides comprises from about 8 to about 30 nucleotide units, and wherein each of said one or more oligonucleotides specifically hybridizes to a target nucleic acid, wherein said target nucleic acid is a DNA or mRNA molecule encoding a mutant or wildtype ras protein, and wherein said one or more  
10 oligonucleotides are entrapped within liposomes.
2. The composition of claim 1, wherein at least one nucleotide of at least one of said one or more oligonucleotides is modified at the 2' position of the sugar portion of said nucleotide.
- 15 3. The composition of claim 1, wherein at least one of said one or more oligonucleotides is a chimeric oligonucleotide having a first region, wherein said first region has at least one nucleotide modified to enhance the affinity of said oligonucleotide for said target nucleic acid, and a second  
20 region, wherein said second region forms a substrate for RNase H when hybridized to said target nucleic acid.
4. The composition of claim 3, wherein said nucleotide modified to enhance the affinity of said oligonucleotide for said target nucleic acid is modified at the 2' position of the  
25 sugar portion of said nucleotide.
5. The composition of claim 4, wherein said nucleotide modified to enhance the affinity of said oligonucleotide for said target nucleic acid has an alkoxy, alkoxyalkoxy or fluoro substituent at the 2' position.

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6. The composition of claim 3, wherein said second region that forms a substrate for RNase H when hybridized to said target nucleic acid comprises at least one 2'-deoxynucleotide.

5 7. The composition of claim 1, wherein at least one of said one or more oligonucleotides has one or more phosphorothioate linkages.

8. The composition of claim 1, wherein at least one of said one or more oligonucleotides is a peptide nucleic acid.

10 9. The composition of claim 1, wherein at least one of said one or more oligonucleotides is a chimeric oligonucleotide having a first flanking region, wherein said first flanking region has at least one nucleotide modified to enhance the affinity of said oligonucleotide for said target nucleic acid,  
15 a central region, wherein said central region forms a substrate for RNase H when hybridized to said target nucleic acid, and a second flanking region, wherein said second flanking region has at least one nucleotide modified to enhance the affinity of said oligonucleotide for said target nucleic acid, and  
20 wherein said nucleotides modified to enhance the affinity of said oligonucleotide for said target nucleic acid in said first flanking region and said second flanking region can be the same or different.

10. The composition of claim 9, wherein at least one of  
25 said nucleotides modified to enhance the affinity of said oligonucleotide for said target nucleic acid is modified at the 2' position of the sugar portion of said nucleotide.

11. The composition of claim 10, wherein said nucleotide modified to enhance the affinity of said oligonucleotide for

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said target nucleic acid has an alkoxy, alkoxyalkoxy or fluoro substituent at the 2' position.

12. The composition of claim 9, wherein said central region that forms a substrate for RNase H when hybridized to  
5 said target nucleic acid comprises at least one 2'-deoxynucleotide.

13. The composition of claim 9, wherein at least one of said one or more oligonucleotides has one or more phosphorothioate linkages.

10 14. The composition of claim 1, wherein at least one of said oligonucleotides is specifically hybridizable with a translation initiation site or codon 12 of the ras gene.

15 15. The composition of claim 1, wherein one of said oligonucleotides is ISIS 2503 having the sequence of SEQ ID NO:  
2.

16. The composition of claim 1, wherein said liposomes are sterically stabilized liposomes.

17. The composition of claim 16, wherein said sterically stabilized liposomes have a mean particle size of from about  
20 90 to about 110 nm.

18. The composition of claim 16, wherein said sterically stabilized liposomes comprise at least one underivatized vesicle-forming lipid and at least one vesicle-forming lipid which is derivatized with a hydrophilic polymer.

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19. The composition of claim 18, wherein said hydrophilic polymer is a moiety comprising polyethylene glycol.

20. The composition of claim 18, wherein said hydrophilic polymer comprises from about 1 mol% to about 20 mol% of the  
5 total lipid content of the liposomes.

21. The composition of claim 16, wherein said sterically stabilized liposomes comprise at least one underivatized vesicle-forming lipid and at least one vesicle-forming phospholipid having an amino group.

10 22. The composition of claim 21, wherein said vesicle-forming phospholipid having an amino group is selected from the group consisting of phosphatidylethanolamine and distearoyl phosphatidylethanolamine.

23. The composition of claim 16, wherein said sterically  
15 stabilized liposomes comprise at least one sterol.

24. The composition of claim 23, wherein said sterol is cholesterol.

25. The composition of claim 1, wherein said liposomes  
20 comprise at least one lipid having two hydrocarbon chains and a polar head.

26. The composition of claim 25, wherein said lipid having two hydrocarbon chains and a polar head is selected from the group consisting of phosphatidylcholine, distearoyl phosphatidylcholine and dipalmitoylphosphatidylcholine.

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27. The composition of claim 16, wherein said sterically stabilized liposomes comprise at least one glycolipid.

28. The composition of claim 27, wherein said glycolipid is ganglioside GM<sub>1</sub> or sphingomyelin.

5        29. A method of modulating the expression of a ras gene comprising contacting cells, tissues, organs or organisms expressing said ras gene with the composition of claim 1.

30. A method of inhibiting the growth of cells comprising contacting said cells with the composition of claim 1.

10       31. The method of claim 30, wherein said ras gene is a human ras gene.

32. The method of claim 31, wherein said human ras gene is H-ras, K-ras or N-ras.

15       33. A pharmaceutical composition comprising the composition of claim 1, wherein said ras gene is a mammalian ras gene.

34. A pharmaceutical composition comprising the composition of claim 1, wherein said ras gene is a human ras gene.

20       35. The pharmaceutical composition of claim 34, wherein said human ras gene is H-ras, K-ras or N-ras.

36. A pharmaceutical composition comprising (a) the composition of claim 1 in combination with (b) one or more

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chemotherapeutic agents which do not act by an antisense mechanism.

37. The pharmaceutical composition of claim 36 in which said one or more chemotherapeutic agents is or are entrapped  
5 within sterically stabilized liposomes.

38. A pharmaceutical composition comprising the composition of claim 1 in combination with one or more non-ras-targeted oligonucleotides, wherein each of said one or more non-ras-targeted oligonucleotides comprises from about 8 to  
10 about 30 nucleotide units, and wherein each of said one or more non-ras-targeted oligonucleotides specifically hybridizes to a target nucleic acid, wherein said target nucleic acid is a DNA or mRNA molecule deriving from a mammalian mutant or wildtype cancer associated gene other than a ras gene.

15 39. The pharmaceutical composition of claim 38, wherein said one or more non-ras-targeted oligonucleotides is or are entrapped within sterically stabilized liposomes.

20 40. A method of treating or preventing a condition arising from the activation of a ras gene comprising administering to a mammal having said condition a therapeutically or prophylactically effective amount of the pharmaceutical composition of claim 33.

25 41. A method of reducing the rate of growth of a tumor or cancer in a mammal comprising administering to a mammal having a tumor or cancer a therapeutically or prophylactically effective amount of the pharmaceutical composition of claim 33.

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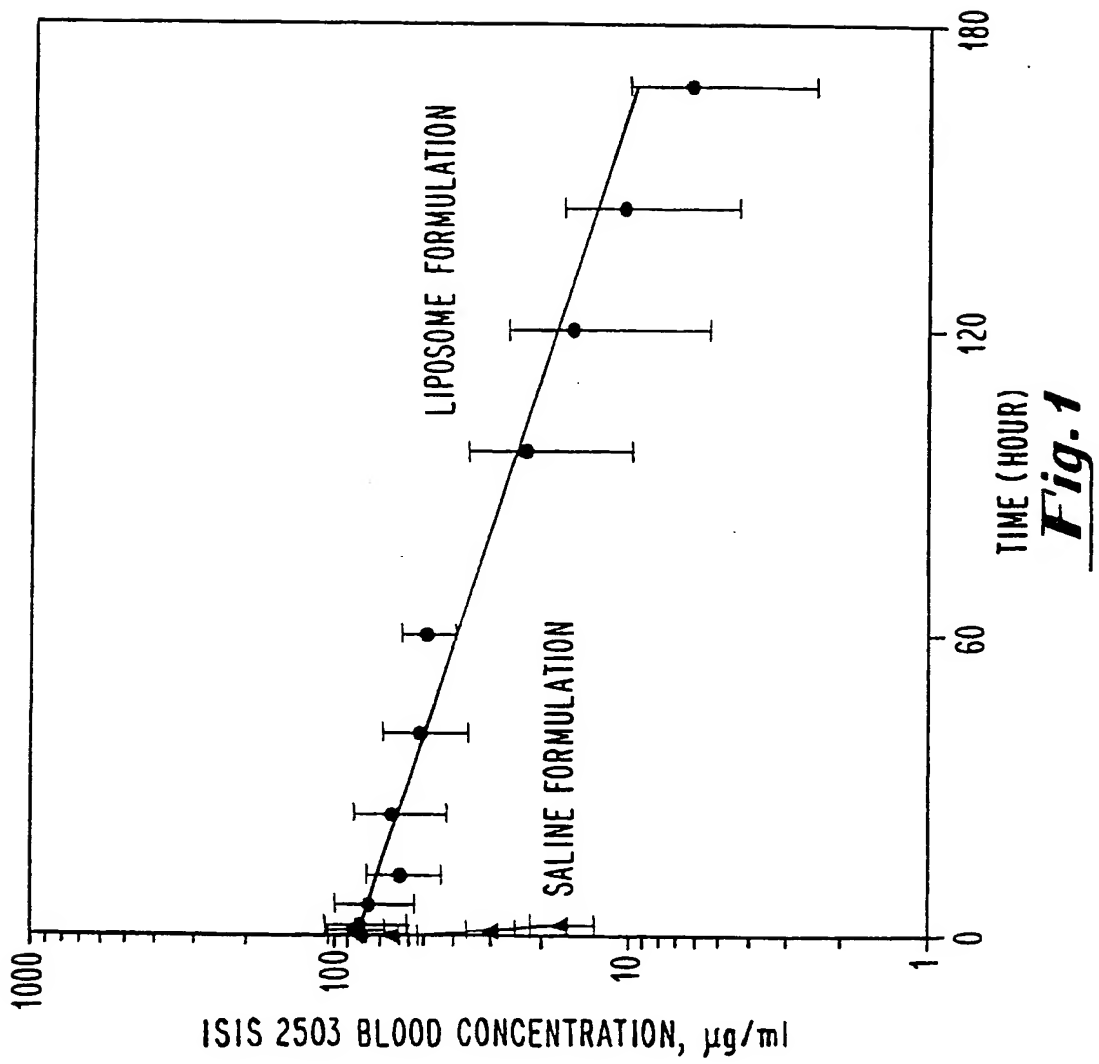
42. A method of treating or preventing cancer in a mammal comprising administering to said mammal a therapeutically or prophylactically effective amount of the pharmaceutical composition of claim 33.

5        43. A method of treating or preventing cancer in a mammal comprising administering to said mammal a therapeutically or prophylactically effective amount of the pharmaceutical composition of claim 33.

44. The method of claim 40, wherein said mammal is a  
10 human.

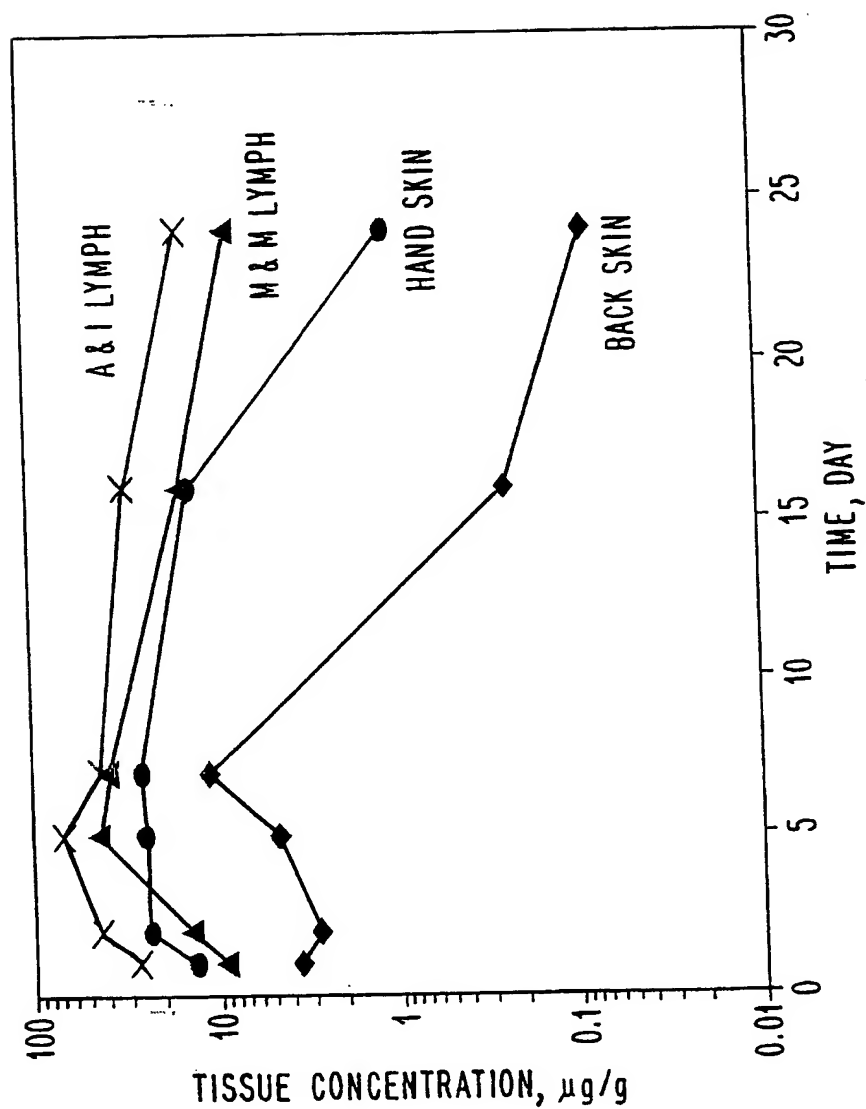
45. A pharmaceutical composition comprising the composition of claim 15.

1/7



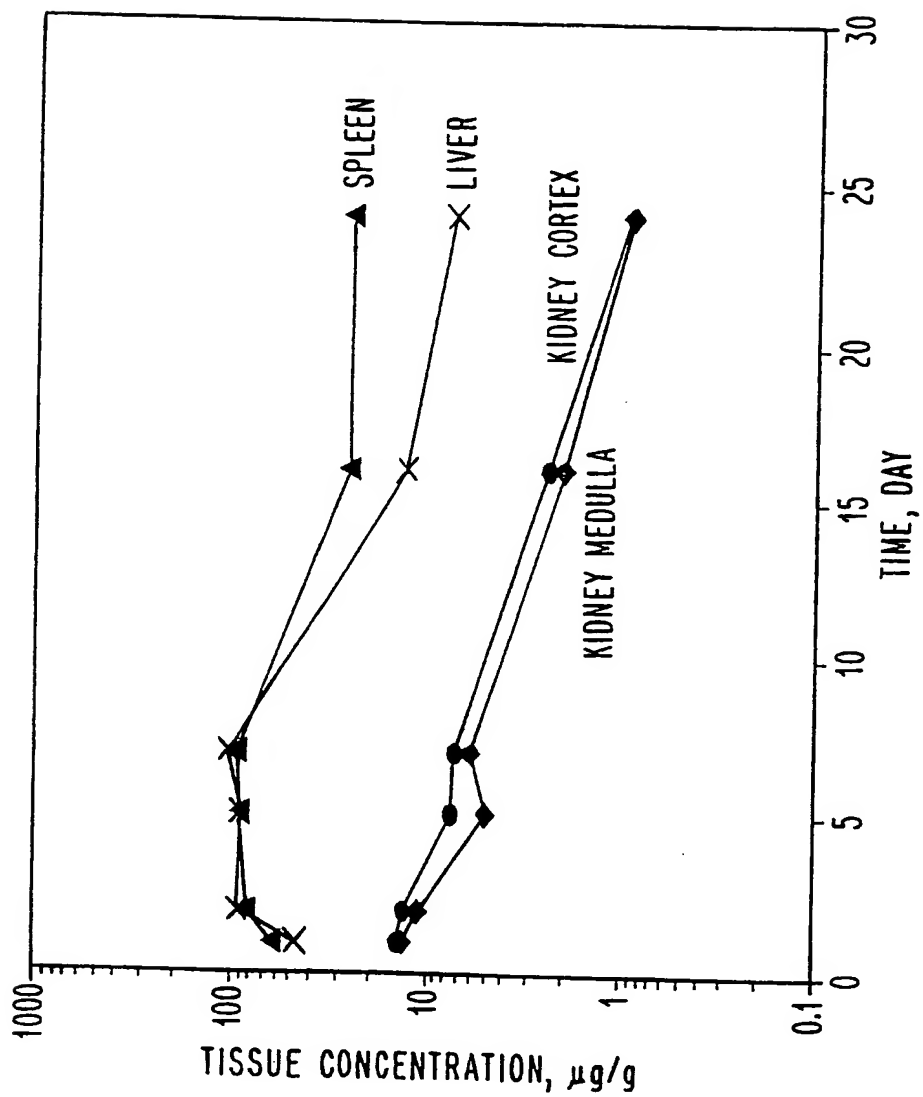


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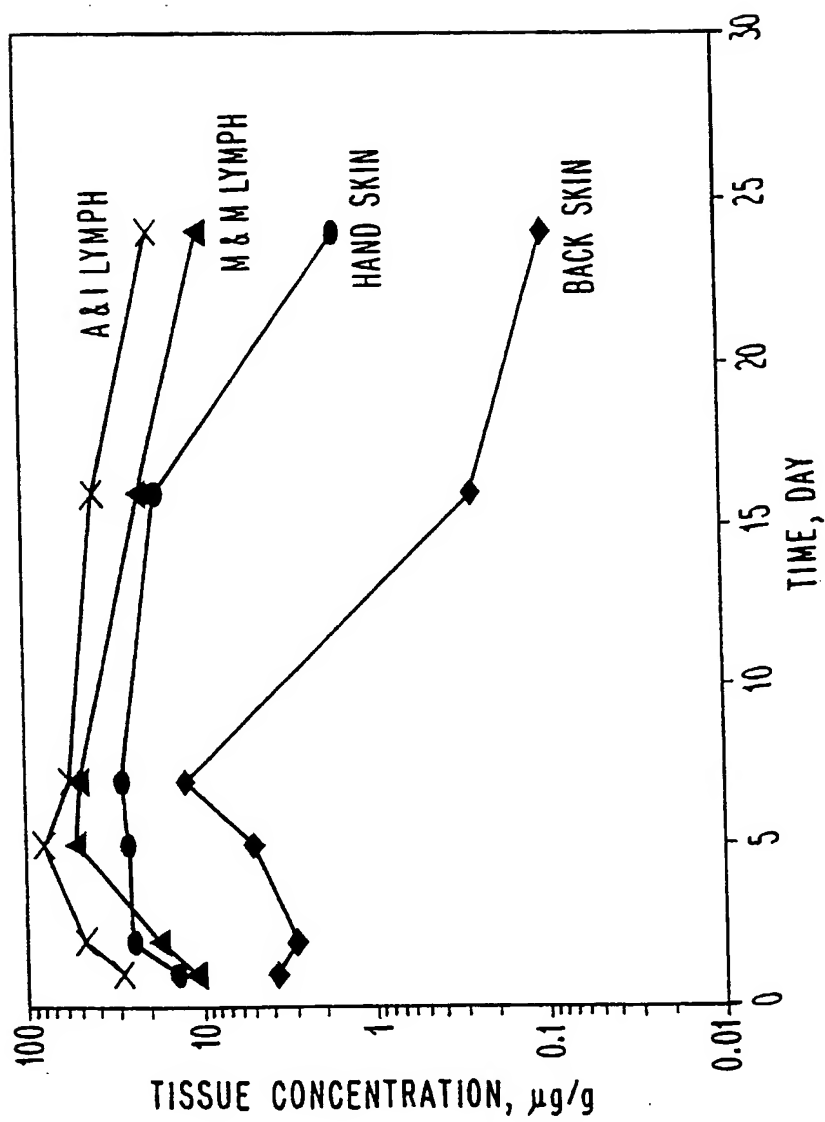


**Fig. 2**

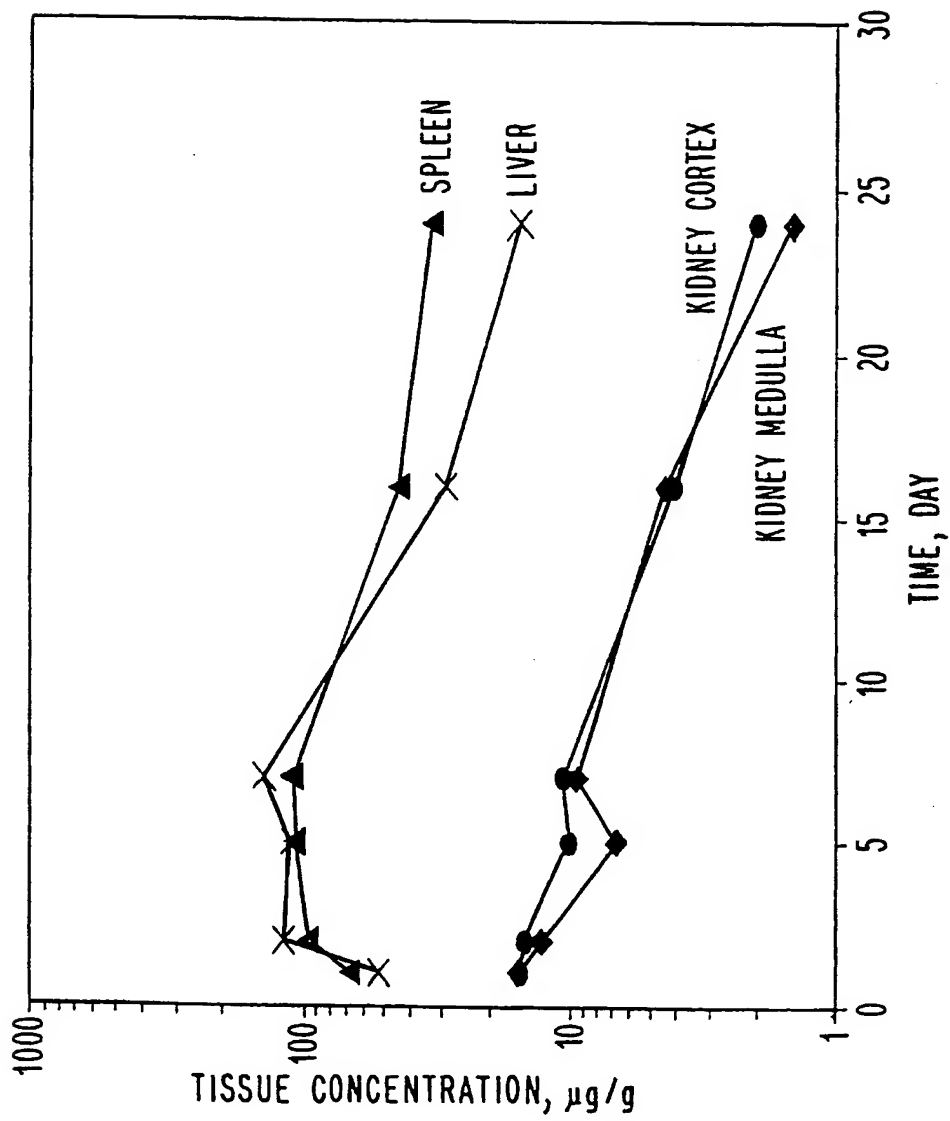
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*Fig. 3*

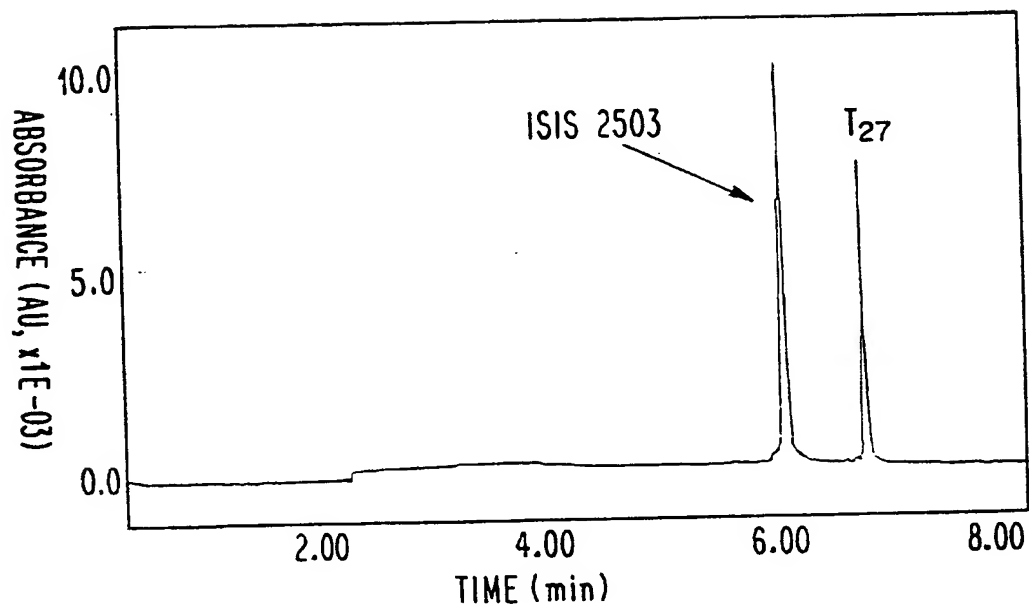
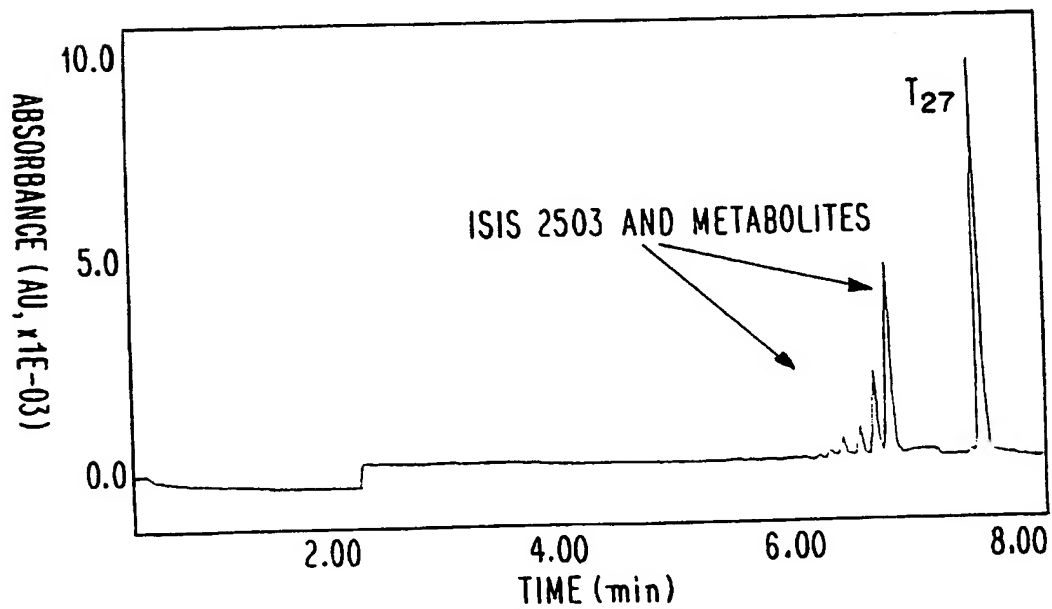
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***Fig. 4***

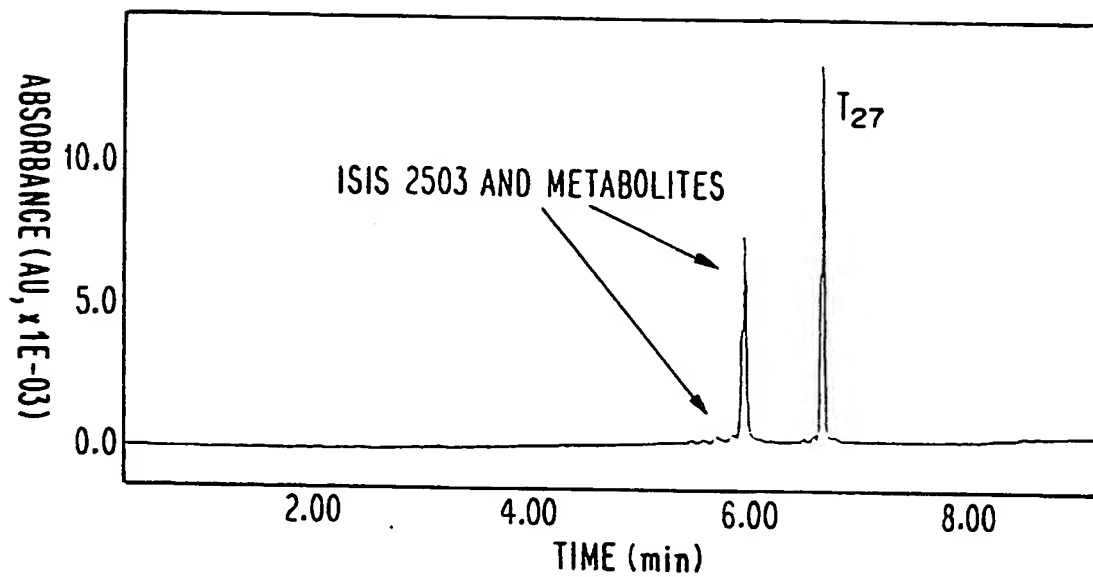
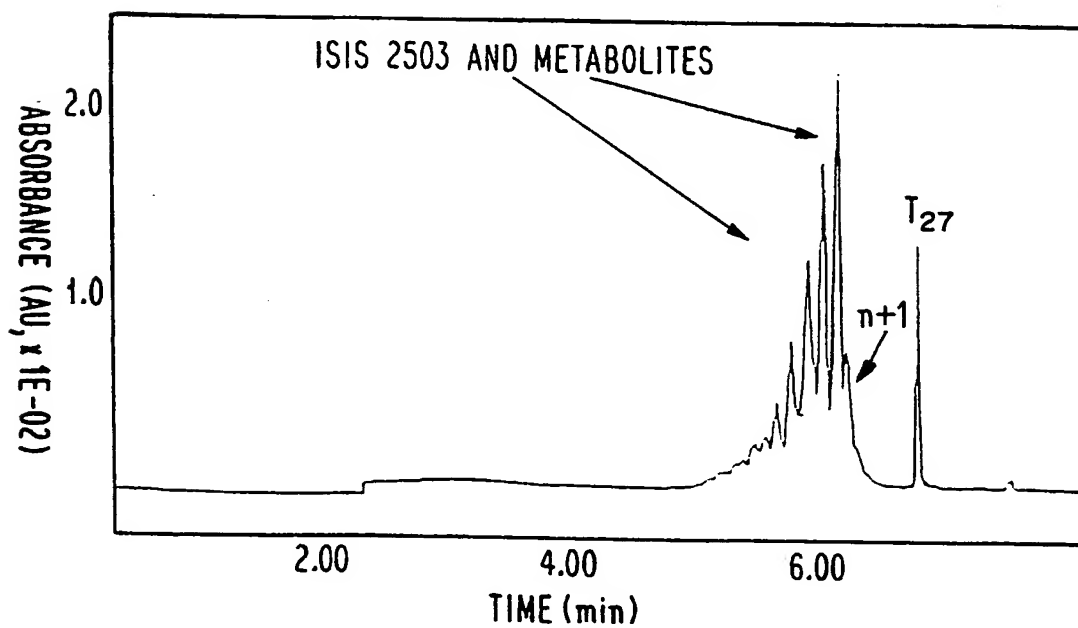
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*Fig. 5*

6/7

***Fig. 6A******Fig. 6B***

7/7

***Fig. 7A******Fig. 7B***

## SEQUENCE LISTING

<110> Hardee, Gregory E.  
Geary, Richard S.  
Levin, Arthur  
Templin, Michael V.  
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<120> Liposomal Oligonucleotide Compositions for Modulating  
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18

<210> 17

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<210> 18

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<210> 19

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<210> 20

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<210> 32

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<210> 39

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<210> 41

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## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US98/22821

<b>A. CLASSIFICATION OF SUBJECT MATTER</b> IPC(6) : A61K 48/00; C07H 21/04, 21/02; C12N 15/11 US CL : 536/24.1, 24.5; 435/375; 514/44 According to International Patent Classification (IPC) or to both national classification and IPC		
<b>B. FIELDS SEARCHED</b> Minimum documentation searched (classification system followed by classification symbols) U.S. : 536/24.1, 24.5; 435/375; 514/44 Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched NONE Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) APS, MEDLINE, BIOSIS, SCISEARCH, EMBASE, CAPUS		
<b>C. DOCUMENTS CONSIDERED TO BE RELEVANT</b>		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	CHANG et al. Antisense Inhibition of ras p21 Expression that is Sensitive to a Point Mutation. Biochemistry, 27 August 1991 Vol. 30, No. 34, pages 8283-8286, see entire document.	1, 2, 7, 14, 17-27
Y	CHONN et al. Recent Advances in Liposomal Drug-Delivery Systems. Current Biology, 1995. Vol. 6, pages 698-708, see entire document.	1-32, 45
Y	US 5,576,208 A (MONIA et al.) 19 November 1996, see entire document.	1-32, 45
Y	UHLMANN et al. Antisense Oligonucleotides: A New Therapeutic Principle. Chemical Reviews June 1990. Vol. 90, No. 4, pages 543-584, see entire document.	1, 2, 7, 14, 17-32
<input type="checkbox"/> Further documents are listed in the continuation of Box C. <input type="checkbox"/> See patent family annex.		
* Special categories of cited documents: *A* document defining the general state of the art which is not considered to be of particular relevance *E* earlier document published on or after the international filing date *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) *O* document referring to an oral disclosure, use, exhibition or other means *P* document published prior to the international filing date but later than the priority date claimed *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art *&* document member of the same patent family		
Date of the actual completion of the international search 17 DECEMBER 1998		Date of mailing of the international search report 08 FEB 1999
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230		Authorized officer: SEAN MCGARRY Telephone No. (703) 308-0196